

**THE MECHANISMS OF WOOL DUST-MEDIATED INFLAMMATION
AND
LEUKOCYTE ACTIVATION IN THE RAT LUNG.**

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I declare that this thesis was written by me and that the work contained herein is my own.

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ABSTRACT.

The purpose of this study was to try to explain, using a rat model, the symptoms of bronchitis reported by some members of the workforce in wool textile mills in the north of England and in grain handlers. Inflammation was evident in rats following intratracheal instillation of dust collected from the air of wool mills and grain dusts. The inflammation could arise from at least four possible pathways and so we investigated each of these.

1. Direct toxicity towards airspace epithelial cells or alveolar macrophages.

There was no significant toxicity of wool or grain dust toward either cells of a human alveolar epithelial cell line (A549), or rat alveolar macrophages.

2. Activation of adhesion molecules on leukocytes.

A constant finding in the lungs from wool and grain-treated animals was aggregates of mononuclear cells which were almost entirely macrophages. It was hypothesized that prolonged up-regulation of adhesion molecules could account for aggregate formation, which may be mediated through the action of bacterial endotoxin present on the dusts, and which could enhance inflammation. Macrophage aggregation could be produced *in vitro* after stimulation, and antagonists of adhesion molecule activation pathways abolished the formation of aggregates.

3. Secretion of pro-inflammatory cytokines by dust-exposed alveolar macrophages.

Tumor Necrosis Factor (TNF) was secreted by alveolar macrophages after treatment with wool and grain dusts *in vitro*. Additionally, bacterial endotoxin which we detected on the dusts and which was present in leachates of dust, was shown to play an important role. Depletion of endotoxin in dust leachates resulted in substantially less TNF being released.

4. Immune responses to organic antigens in the wool dust.

The involvement of endotoxin in causing local activation of the immune system was assessed by measuring proliferation of lung lymph node lymphocytes from animals instilled with dusts, dust leachates and endotoxin. There was increased thymidine uptake in lymph node lymphocytes from treated animals, which suggested stimulation or priming either from the direct action of endotoxin leached from the

surface of the dusts or through TNF released from activated macrophages. These studies confirm that dust collected from the air of wool mills, and grain dust have the ability to produce inflammation via several different pathways, and that these effects may be mediated in a large part through the action of bacterial endotoxin present in the dusts.

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INTRODUCTION.

THE MECHANISMS OF WOOL DUST-MEDIATED INFLAMMATION AND LEUKOCYTE ACTIVATION IN THE RAT LUNG.

INTRODUCTION.

BACKGROUND TO THE STUDY.

1.1.1. IOM Studies on Respiratory Symptoms in Wool Workers.

A previous epidemiological study carried out by the Institute of Occupational Medicine (IOM) investigated exposure to dust in wool textile mills and the resulting respiratory symptoms produced in the workers (Love et al, 1988; Love et al 1991). The survey was carried out by collecting information from employees working in mills in West Yorkshire by questionnaire, regarding smoking history, previous occupation, and common respiratory symptoms such as cough, phlegm, wheeze, chest tightness, shortness of breath, rhinitis and nosebleeds.

Airborne dust sampling revealed levels ranging from zero to over 10mg/m^3 and that high concentrations of dust were associated with certain jobs in the wool production process. In addition, endotoxin which has potent pharmacological and immunological effects, and is produced by contaminating bacteria on the surface of the organic dust, were on occasion very high. The dust sampling instrument sampled dust which was inspirable, that is dust which can pass into the nose and mouth. (Mark et al, 1985) This also includes the portion of dust which is respirable, and can pass into the bronchial and alveolar regions of the lung.

The study carried out by the IOM showed that symptoms were related to dust exposure, confirming the findings of other wool dust studies (Zuskin et al, 1976; Allardice et al, 1983). Further confirmation of an occupational cause to these respiratory symptoms was present in the reported improvement in symptoms when workers were absent from the workplace, for example at weekends or holidays (Love et al, 1988). It was also clear that the dust affected all levels of the respiratory tract, producing rhinitis, chronic bronchitis, and breathlessness (Love et al, 1988). This suggested that the dust may produce its effects through a common irritative mechanism, which could have an immunological/inflammatory basis involving the epithelium in the airways and nose.

The composition of the wool dust and contaminants associated with it are likely to influence the response of the lung. The dust sampled in this study was almost

entirely wool, although contaminating microbes, endotoxin, dyes and other organic material could be detected (Love et al, 1988; Donaldson et al, 1990). An interesting observation was that the dust produced more symptoms and was more harmful in the less processed states, in the early stages of the wool production process (Love et al 1988), and there was a greater reduction in FEV₁ in dyers and wool scourers (Love et al, 1991). This suggested involvement of chemicals such as caustic soda, which is used in the scouring process, and dyes.

1.1.2. Prevalence of Symptoms in Wool Workers.

The 2153 workers who were examined in this study described the symptoms listed in Table 1.

SYMPTOM	FREQUENCY
Persistent Cough and Phlegm (Chronic Bronchitis)	9%
Wheeze	31%
Breathlessness	10%
Variable Breathlessness	3%
Persistent Rhinitis	18%
Persistent Conjunctivitis	10%
Persistent Chills	2%
>10 Nosebleeds/Year	2%

Table 1. Symptoms reported by the workforce in wool textile mills.

Rhinitis and conjunctivitis were reported to be worse in the spring and summer months, which suggested an allergic component. In addition, workers complained of symptoms being worse in areas where dust levels were highest (Love et al, 1988; Love et al, 1991). When these symptoms were related to dust concentrations and were adjusted for smoking, age, sex and ethnic background (over 600 workers were Asian), a positive correlation was found (Love et al, 1988). In particular, in

workers who reported variable breathlessness, this was also related to the concentration of wool dust in the air. In workers who smoked, the prevalence of chronic bronchitis was more than three times greater than in non-smokers (Love et al, 1988).

The study concluded that wool dust produced symptoms which suggested inflammation at all levels of the respiratory tract, and were likely to produce functional impairment of the lungs in the form of chronic bronchitis and breathlessness. The symptoms suggested that, at nuisance levels of dust in the factories, harmful effects may result, and that exposure limits could be set to reduce risks to workers. In addition, further research would be necessary to determine the cellular and pathological effects which may be present.

1.1.3. Radiological Appearance and Lung Function.

Radiological changes which could be present in the workforce included evidence of pulmonary fibrosis or extrinsic allergic alveolitis, which is a feature of workers exposed to dusts of organic origin, contaminated by microorganisms. Medical X-ray readers and an independent panel of lay readers found that there was no significant relationships between the airborne concentration of dust in the wool mills and radiographic appearance (Love et al, 1991). Any abnormalities present were in smokers, although it was suggested that workers who had spent six years or more in their current job showed a greater profusion of opacities compared to workers with less than six years in the same job (Love et al 1991).

In the lung function investigation, small effects were observed in certain categories of workers, for example, significant effects were noted for FEV₁/FVC ratio in European women. (Love et al, 1988) The conclusion was that it was not possible to say with certainty that wool mill dust could cause a particular change in a particular lung function index. In jobs not involving exposure to wool dust, for example in the dyeing and scouring processes, significant decreases in FEV₁ were demonstrated when correction was made for confounding factors. An interesting observation was that these findings were not related to asthmatic effects.

Questions arising from the wool dust study (Love et al, 1988) were investigated in a second project. This study consisted of further relating dust levels to respiratory symptoms by monitoring radiological and functional changes in the lungs of the workers (Love et al, 1991). In addition, biological effects were studied by exposing

laboratory rats to the wool dust, and examining the lung tissue and cellular responses (Donaldson et al, 1990).

The findings of these experiments suggested that wool dust could cause injury to epithelial cells of the lung (Donaldson et al, 1990), and that there were non-specific immunostimulatory effects produced by wool dust in a mouse model (Love et al, IOM report, 1988). In addition, further immune effects were suggested from experiments which showed that wool dust instilled into rat lungs caused aggregation of the lavaged broncho-alveolar cells (Donaldson et al, 1990). These results suggested that inflammation and immunological effects produced by wool dust in the rat model could provide the starting point for the symptoms reported by some members of the workforce in wool textile mills.

1.1.4. Experimental Studies.

A recommendation was made by the IOM report (Love et al, IOM report, 1988) to the Health and Safety Executive (HSE) that the findings should be further investigated experimentally. In particular there should be a study on the cellular mechanisms by which wool dust caused inflammation in the lung. This further research was funded by the HSE, and forms the basis of this thesis.

1.2. PULMONARY RESPONSES TO ORGANIC DUST INHALATION.

The previous studies carried out by the IOM showed that there was no wool dust exposure-related asthma present in the workers. Direct toxic/irritative effects, Extrinsic Allergic Alveolitis (EAA) type effects and non-specific endotoxin-mediated cellular modulation were considered likely to be the most important mechanisms at work in wool dust-mediated inflammation.

1.2.1.1. Inhalation of Organic Dusts.

There is a wide range of organic substances which can be inhaled in either particulate or aerosol form and give rise to parenchymal lung disease (Forster et al, 1989). Amongst the substances implicated are vegetable dusts (Pernis et al, 1961), animal proteins, spores (Cox et al, 1988), organic chemicals and microorganisms (Lacey et al, 1980). Allergens associated with organic dusts have been identified as various forms of mould, gram negative bacteria and leachable

products derived from these contaminants (Rylander, 1982; Walsh et al, 1987; Lacey and Crook, 1988).

1.2.1.2. Exposure to Cotton Dust.

Cotton dust-related lung disease (Byssinosis) has been extensively studied in view of the large population of exposed workers. Byssinosis manifests as acute dyspnoea, with cough and reversible breathlessness on one or more days of a working week (Chinn et al, 1976; Vuk-Pavlovic et al, 1988). The symptoms are usually worse on the Monday morning, when workers return to work after a week-end. In severe cases, permanent respiratory disability due to irreversible airflow obstruction can occur (Haglund et al, 1983). Byssinosis in the cotton industry is related to the airborne concentration of dust, (Haglund and Rylander, 1984), and the causative agent is believed to be contaminating endotoxin rather than the cotton fibres themselves (Rylander et al, 1979; Rylander, 1982). A clinical feature of this disease is the fall in FEV₁ which has been shown to be related to increased numbers of bacteria on the cotton dust (Rylander et al, 1979). In some cases, changes in lung function are accompanied by fever, which is also considered to be due to endotoxin.

1.2.1.3. Exposure to Grain Dust.

Grain dust is produced during the processes of harvesting, milling, transport or storage of barley, wheat, oats maize and rye. The dust itself is a heterogeneous mixture of components from the grain plant for example pericarp, endosperm and starch granules (Chan-Yeung et al, 1987). As with other organic dusts, particular importance is placed on the presence of contaminating endotoxins and aflatoxins produced by fungal spores (Baxter et al, 1981). In addition, leachable products from the dust itself as well as contaminants from rodents and mites that grow during grain storage may play a role (Leskinen and Klem, 1987). One of the clinical symptoms associated with exposure to grain dust is grain fever, which as the name suggests, results in raised temperature, headache, malaise, muscular and chest pain, dyspnoea and cough (Darke et al, 1976). Several studies have confirmed that exposure to grain dust in the long term results in chronic bronchitis and chronic obstructive pulmonary disease (Chan Yeung et al, 1978; Becklake et al, 1980).

1.2.1.4. Exposure to Wool Dust.

There are few reports dealing exclusively with the exposure of workers to wool dust. It has been suggested that there is a considerably increased prevalence of non-

specific bronchopulmonary disease in workers who have been employed in the wool industry for long periods of time (Jordeczka et al, 1970). Other workers found that the development of chronic bronchitis and emphysema was related to the concentration of wool dust (Brysiewicz et al, 1970; Love et al, 1988; Love et al, 1991), and the duration of employment. As with cotton and grain exposure, inhalation of wool dust can result in decrements in lung function, in particular FEV₁, although there were no typical cases of byssinosis in these studies (Zuskin et al, 1976). In other stages in the wool production process, in particular the dyeing and scouring process, different chemicals are used which have been implicated in asthmatic symptoms and poor lung function in some workers (Love et al, 1991). In other workers, symptoms suggestive of humidifier fever rather than exposure to wool dust have been reported (Love et al, 1988).

1.2.1.5. Other Dusts.

The organic nature of animal fur or hair renders it antigenic when inhaled into the lungs. The result of this is likely to be the production of an immune response, which can affect all regions of the respiratory tract. Work previously described (Pimentel, 1970), showed the effects in a worker exposed to fox fur. The pathology of the lung showed the presence of granulomatous lesions, a characteristic feature of an immunologically-related process (James, 1991). In addition, hair was present in these lesions, giving further evidence of the involvement of organic dust in this type of disease.

1.2.2. Extrinsic Allergic Alveolitis (EAA).

In Extrinsic Allergic Alveolitis (EAA), type 3 hypersensitivity reactions may occur in early disease whilst cell-mediated effects predominate in the established condition (Skea et al, 1988). The classical pathobiology of EAA involves the formation of a granulomatous lesion, due to inflammation produced by inhaling organic material (Hughes and Haslam, 1990). These patients have increased numbers of lymphocytes which infiltrate the alveolar walls, and the cells are in an activated state (Mornex et al, 1984). Occasionally, antibodies to the inhaled antigen can be detected in the blood (Fink, 1984). Immunoglobulin (IgM and IgG) can form complexes and precipitate antigen in sensitised individuals. These interactions may occur in the interstitium of the lung where the complement system may become activated with the recruitment of neutrophils. Continued exposure to the sensitizing antigen results in chronic interstitial inflammation and fibrosis (Kaltreider, 1973) (see section 1.2.5).

1.2.3. Complement.

Complement is a group of proteins designated C1-C9 which is present in serum and other body fluids including lung lining fluid. The system can be activated by antigen/antibody complexes, via the so called classical pathway or by diverse substances such as endotoxin, complex polysaccharides or coagulation factors via the 'alternative' pathway (Olenchok et al, 1978; Wilson et al, 1980). Complement is important in host defence because the opsonisation and lysis of bacteria often depend on its presence (Cooper and Nemerow, 1985).

Complement becomes activated by a series of pro-enzymes, each activating the next in a complex cascade-like fashion. Component C3 is the most abundant factor and its cleavage in the rate-limiting step results in activation. If the cascade goes to completion, assembly of the later components C5-C9 in membranes eventually produce lysis (Müller-Eberhard, 1985). In addition, splitting of C3 and C5 produces fragments C5a and C3a (anaphylatoxins) which play a major pro-inflammatory role (Burger, 1987). Both C3a and C5a are chemotactic for macrophages and neutrophils (PMN) and can release histamine from mast cells and eosinophils resulting in contraction of smooth muscle cells in blood vessels and airways (Warren and Holford-Strevens, 1986). C3b is particularly important as an opsonin which, when present on the surface of bacteria promotes their phagocytosis. Macrophages and PMN have receptors for C3b on their surface and can more readily phagocytose bacteria or particles which have become opsonised in this way (Lambris, 1988).

1.2.4. Granulomatous Inflammation.

This type of inflammation is caused by several organic dusts and results from chronic inflammation where there is persistence of the stimulus, tissue destruction and fibrosis (Haschek and Witschi, 1979). The characteristic lesion produced is the granuloma which is comprised of various cell types including macrophages, and giant cells and epithelioid cells, lymphocytes, and fibroblasts (Garrett et al, 1984; Kunkel et al, 1989).

Two types of granulomatous inflammation have been identified, immunologic (hypersensitivity-type) or non-immunologic (foreign body type), (Kunkel et al, 1989; James, 1991). The former is the result of a delayed antigen specific immunologic response and can be produced by a variety of persistent stimuli including bacteria and fungi (Salvaggio and Karr, 1979). The foreign body type

lesion is not the result of an antigen-specific response and may be relatively inactive. In animal studies with grain dust, granulomatous nodules have been reported (Friborsky et al, 1972). The development and maintenance of the lesion is brought about by the action of cytokines especially Interleukin-1 (IL-1) and Tumor Necrosis Factor (TNF), which are secreted by the macrophages, lymphocytes and monocytes (Kasahara et al, 1989; Chensue et al, 1989).

1.2.5. Pulmonary Fibrosis.

Some patients with EAA caused by inhaling organic dusts and mediated by T-cell hyperreactivity, may progress to develop interstitial lung fibrosis (Fink, 1984). Fibrosis is a chronic disease in man which results in decreased diffusion capacity of oxygen into the capillaries because the interstitium is thickened due to deranged collagen (Crystal et al, 1976). The fibroblast is central to the pathogenesis of fibrotic lung disease and in this regard, the turnover of lung collagen could be important in the regulation of collagen deposition. The importance of cytokines in the regulation of collagen synthesis has been described (Schmidt et al, 1982; Freundlich et al, 1986). Some disorders which are thought to be immunologically-mediated, such as granuloma formation, are associated with increased lymphokine and cytokine secretion which enhance fibroblast function (Keogh and Crystal, 1981).

A potent fibrogenic cytokine is platelet-derived growth factor (PDGF) which is secreted by cells including smooth muscle, endothelial cells and activated macrophages and which has been implicated in regulating connective tissue production and remodelling (Freundlich et al, 1986; Bonner and Brody, 1991). Transforming growth factor- β (TGF- β) is a cytokine which has been well characterized and which has been shown to increase collagen synthesis in human lung fibroblasts (Freundlich et al, 1986). Pulmonary fibrosis is considered to result from the failure of normal tissue to repair after injury (Hance and Crystal, 1975; Fulmer and Crystal, 1976) and these effects may be important in organic dust-related lung injury.

1.2.6. The Role of Endotoxin in Organic Dust-Mediated Lung Responses.

Gram negative bacteria and their components or products are potent activators of macrophages (Rylander and Snella, 1983). Endotoxin or lipopolysaccharide (LPS), forms part of the outer membrane of these bacteria, and is a ubiquitous substance (Rietschel and Brade, 1992) which is found as a contaminant on dusts, and in the air

(Rylander and Lundholm 1978; Rylander et al, 1979; Olenchock et al, 1980). Cell responses to LPS exposure *in vitro* include the secretion of pro-inflammatory cytokines, oxygen radicals and upregulation of cell adhesion molecules (Meyer et al, 1991). In addition, substances such as TNF can 'prime' macrophages and neutrophils into such a state that LPS may produce an enhanced response (Sun et al, 1990).

Activation of complement via the alternative pathway is another effect produced by LPS, as described above, which promotes inflammation. Inhaled endotoxin depositing in alveolar lining fluid may cause complement to become activated (Wilson et al, 1980). Alveolar macrophages may be directly stimulated by LPS or via activated complement components, to produce cytokines and chemoattractant factors which recruit other inflammatory cells. Inflammatory narrowing of the airways or inflammatory thickening of the alveolar septa may cause decreased lung function (Vuk-Pavlovic and Rohrbach, 1990; Milton et al, 1990). In view of the potent stimulating ability and the ubiquitous nature of LPS, this substance alone could account for macrophage and neutrophil activation, inflammation and hence the symptoms produced in the lungs of workers who inhale organic dusts (Rylander and Lundholm, 1978; Rylander, 1987).

1.3. NORMAL LUNG DEFENCE.

1.3.1. Muco-ciliary Escalator (MCE).

The lining epithelium of the conducting airways is composed of a variety of cell types. Of these, the two main epithelial cell types are the ciliated and secretory cells (Sleigh et al, 1988). The latter secrete mucus which forms a discontinuous raft overlying the ciliated cells; the latter cells move the mucus upwards and out of the respiratory tract (Sleigh et al, 1988). Dust depositing directly onto the MCE is trapped in this sticky matrix to be eliminated by the same route. Ciliated cells beat in a synchronized manner, interact with the mucus and periciliary fluid, moving the mucus upwards through the trachea and is eventually swallowed. The importance of this method of particle clearance is shown in diseases such as bronchitis (Iravani and van As, 1972), asthma and in smokers (Sleigh, 1984), where ciliary dyskinesia occurs, resulting in accumulation of mucus and reduction of airway calibre.

On first entering the lung, air passes through the nasopharynx where it is moistened and warmed and also filtered by hairs (Phalen and Oldham, 1983). The conducting airways, consisting of the trachea which branches into two bronchi serving each lung, conveys the air into the respiratory zone of the lung where gas exchange can occur (Burri, 1985). In the distal airways, the MCE defence mechanism is absent, and clearance of small particles and microorganisms relies on phagocytic cells, the alveolar macrophages (Rappolee and Werb, 1989; Keshav et al, 1990; Holian and Scheule, 1990) and polymorphonuclear neutrophilic leukocytes (PMN) (Baggiolini and Dewald, 1985; Ricevati and Mazzone, 1989), and to a large extent on the products they can produce. The majority of macrophages which have phagocytosed particulate material and bacteria find their way onto the MCE, to be finally removed from the airways. Although the function of alveolar macrophages is primarily protective, they have been implicated in producing and perpetuating acute and chronic conditions through the release of mediators (Tate and Repine, 1983; Fujisawa et al, 1990; Richards et al, 1991).

1.3.2. Bronchus-Associated Lymphoid Tissue (BALT).

The airways have areas of organized lymphoid tissue, BALT (bronchus-associated lymphoid tissue), which is important in immunity (Bienenstock, 1984). This tissue is distinct from lymph nodes and is found within the bronchial and bronchiolar walls beneath the epithelium. Microscopically, the epithelium which covers the BALT is distinctly different from airway epithelium in that it is non-ciliated and lacks goblet cells. Follicles of BALT contain T and B-lymphocytes as well as macrophages, which indicates that antigen processing and the initiation of an immune response is possible (Chamberlain et al, 1973).

It has been suggested that alveolar macrophages can phagocytose particulate material in the alveoli and then migrate to BALT (Lipscomb et al, 1982). Other studies have further suggested that antigen may be transported to the lymph nodes from BALT by alveolar macrophages for further immune processing (Racz et al, 1978). An important function of BALT is in the production of IgA (Sminia et al, 1989), an immunoglobulin postulated to play a vital role in maintaining the sterility of the airway mucosa.

1.3.3. The Role of the Lymph Nodes.

Antigen which is deposited in areas of the lung where access to clearance by the mucociliary escalator is not possible, may be cleared by macrophages, via the

lymphatics to the lung lymph nodes. These are situated in the mediastinum. The fate of deposited particles has been followed by intratracheally instilling fluorescent beads into the lungs of dogs (Harmsen et al, 1985). This study showed that, by instilling different coloured beads into the same lung lobe but different areas, tracheobronchial lymph nodes contained macrophages with one or other of the bead colours, but rarely both. This suggested that particles were not translocated passively to the lymph nodes but were carried to the nodes by the macrophages. This tends to confirm that macrophages have a critical role in the development of lung immunity. In a separate confirmatory study (Corry et al, 1984), radio-labelled broncho-alveolar macrophages were instilled into the lungs of guinea pigs and, subsequently, labelled macrophages were detected in hilar lymph nodes. After stimulation by antigen, immune cells can also translocate from lymph node to the lung, (Spencer and Hall, 1984) resulting in immune responses in the lung.

1.3.4. Lung Lining Fluid.

Lung lining fluid, also described as surface active material or surfactant, is a phospholipid-rich lipoprotein which is located on the alveolar surface. Its function is to prevent collapse of the alveolar air spaces by lowering the surface tension at the air tissue interface (Ansfield et al, 1979). Surfactant is synthesized and secreted by the alveolar type 2 epithelial cells and contains potent inhibitors of lymphocyte proliferation (*in vitro*) (Ansfield et al, 1979), complement components and proteinase inhibitors (Rooney, 1985). Inhaled antigen may find its way to the alveoli and interact with macrophages and lymphocytes to initiate an immune response. The function and suppressive action of surfactant may be to regulate an immune response which may be potentially damaging to the gas exchange surfaces of the alveoli (Wilsher et al, 1988).

1.3.5. Alveolar Macrophages.

These cells are derived from the circulating blood monocytes and represent the majority of cells found in the lower respiratory tract in man (Crapo et al, 1982; Holian and Scheule, 1990). The technique of broncho-alveolar lavage (BAL) is a valuable tool in the study of the macrophage (Semenzato et al, 1985). Alveolar macrophages differ from other tissue macrophages, for example Kupffer cells in the liver and osteoclasts in bone, in that they are exposed to an aerobic environment. For this reason, alveolar macrophages are likely to exhibit different functional characteristics from interstitial and tissue macrophages (Brain, 1988).

Different sub-populations of macrophages have been identified in the rat lung (Chandler and Fulmer, 1987; Brannen and Chandler, 1988) using BAL. The rat lung population is heterogeneous with regard to morphology and function and cells range in size from 12-40 μ m in diameter. Among the sub-populations, differences in phagocytosis and mediator release have been shown (Chandler and Fulmer, 1987), as well as differences in distribution in different disease states (Shellito et al, 1987). Similar differences have been described in human lung cell populations (Hunninghake et al, 1979; Hunninghake et al, 1981).

In chronic inflammatory lung conditions in the rat, the macrophage population consists mainly of cells which are larger and more mature (Brannen and Chandler, 1988). In acute inflammation, cells are small and more monocytic in appearance, suggesting these are newly recruited to the lung, or that they arise from local proliferation of existing cells (Shellito et al, 1987). Macrophages in the interstitium are not recovered by BAL and are obtained by mincing lung tissue or culturing explants (Warner and Brain, 1990). These cells are not exposed directly to particles unless the particles penetrate the interstitium. Compared with alveolar macrophages, these cells have increased potential for proliferation (Lehnert et al, 1985). Other differences between alveolar and interstitial macrophages have been shown in immune function studies (Weissler et al, 1986; Chitko-McKown et al, 1991), and in secretion of inflammatory mediators.

1.3.6. Macrophage Activation.

Macrophages and other cells may become activated via stimulation of cell surface receptors (Hancock et al, 1987; Tartaglia et al, 1991). One of the most important receptors on the alveolar macrophage surface is the immunoglobulin Fc receptor (Perussia et al, 1987). Binding of antibody which has formed an immune complex with its corresponding antigen is a crucial step in the elimination of foreign material. The receptor for the C3b complement fragment is also found on the macrophage and is important in the phagocytosis of opsonised bacteria (Lambris, 1988).

The cytokines IL-2 and Granulocyte-macrophage colony-stimulating factor (GM-CSF) are important in cell activation and maturation and exert their effect through surface receptors on the macrophage surface (Chen and Lin, 1982; Hancock et al, 1987). Platelet-activating factor and leukotriene B₄ also activate cells through surface receptors (Garcia et al, 1987).

1.3.7. Receptor Activation Mechanisms.

Cell activation occurs when the receptor for a particular ligand becomes occupied by that ligand (Kikkawa and Nishizuka, 1986). Receptor coupling to various second messenger-generating systems can produce a conformational change in membrane bound G protein which then activates adenylate cyclase (Birnbaumer and Brown, 1990). This increases the intracellular concentration of cyclic AMP which in turn activates protein kinase and leads to the cellular response which may be gene expression, degranulation or the production of the respiratory burst.

In a second pathway of cell activation, G protein activates membrane phosphodiesterase, resulting in the conversion of phosphatidylinositol 4,5-bisphosphate to inositol triphosphate and diacyl glycerol (Streb et al, 1983; Berridge and Irvine, 1984). These can then activate protein kinase C, resulting in cell activation. An important method of cell activation depends on the intracellular changes in calcium ion concentration (Hirasawa and Nishizuka, 1985). Inositol triphosphate is important in controlling calcium ion fluxes, and calcium is required for protein phosphorylation (Streb et al, 1983; Berridge and Irvine, 1984; Hughes, 1990).

1.3.8. Phagocytosis

One of the main features of the alveolar macrophage is its ability to phagocytose and remove foreign particulate material from the alveolar region of the lung. Phagocytosis occurs in several distinct steps, which depend on the interaction of cell surface receptors and the particle (Walters and Papadimitriou, 1978; Nathan, 1986). Macrophages and PMN move toward target particles along a gradient of chemotactic molecules. The leading edge of cytoplasm surrounds the particles and fuses to form a phagosome (Adams and Hamilton, 1984). These steps cause the macrophage to undergo changes such as increased protein synthesis, increased size and membrane ruffling and the secretion of various mediators including proteinases, lysosomal enzymes and cytokines (Gordon et al, 1974; Gordon, 1978; Goodman et al, 1982). Hydrolytic enzymes are released into the phagosome to degrade the phagocytosed particles. Particles which cannot be degraded eventually may be removed if the macrophage reaches the mucociliary escalator, or may be carried inside macrophages to the lymph nodes (Harmsen et al, 1985).

Another major defence mechanism associated with phagocytosis and macrophage activation is the production of reactive oxygen intermediates hydrogen peroxide,

superoxide anion and hydroxyl radical (Roos and Balm, 1980). These oxidants are particularly active against bacteria, but can also produce significant injury to bystander cells (Hyslop et al, 1988). In certain circumstances, for example in apoptosis, neutrophils can be phagocytosed by macrophages without the release of mediators (Mangan and Wahl, 1991). Similarly, phagocytosis of the relatively inert dust TiO_2 , causes much less mediator release compared with quartz or long fibre amosite asbestos (Donaldson et al, 1992b).

1.3.9. Transfer of Material to Lymph Nodes.

One of the more important mechanisms for initiation of immune responses in the lung is transport of antigen to the lymph nodes by the alveolar macrophage. Particles reaching the alveoli are phagocytosed by the resident alveolar macrophages, the majority of which eventually find their way onto the MCE. The phagocytosis of dust may also result in toxic effects towards the macrophages, which may die in the alveoli and be phagocytosed by other macrophages (Green and Burlington, 1973). In addition, macrophages may enter the interstitium and migrate into the pulmonary lymphatic system and reach the lymph nodes. The importance of the translocation of particles or antigen to the lymph nodes by macrophages is that antigen processing occurs in the phagocytes followed by presentation at the macrophage surface which is obligatory for initiation of immune responses in the lymph nodes.

Particles can also be endocytosed by the Type-1 pneumocytes, reaching the interstitium where interstitial macrophages phagocytose and carry the particles to the lymphatics and then to the lymph nodes (Sorokin and Brain, 1975; Adamson and Bowden, 1981). Particles have been shown in the interstitium and in the lymph nodes, hours after deposition, suggesting that some particle translocation may occur independent of macrophages (Ferin and Feldstein, 1978). Soluble stimuli, such as the cytokine TNF, produced by activated macrophages, may reach the lymph nodes and cause lymphocyte activation.

1.3.10. The Macrophage Secretory Products - Their Role in Inflammation.

Macrophages secrete a huge range of different bioactive compounds (Papadimitriou and Ashman, 1989). For the purpose of the present discussion, the more important

secretory products of activated macrophages are oxidants, proteinases, and cytokines of various types.

1.3.10.1. Oxygen Radicals.

The role of the macrophage in keeping the alveolar region of the lung sterile is well established. Small particles reaching this region of the lung are dealt with by the macrophage (Nathan and Cohn, 1981). Phagocytosis internalizes particles and bacteria into phagosomes where oxygen radicals such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical are released (Ward et al, 1988). These oxygen species may also be released into the external environment where in some cases tissue injury can ensue (Hyslop et al, 1988). Oxygen-derived molecules such as hypochlorous acid and hydroxyl radical generated through the hydrogen peroxide-myeloperoxidase system are the most bactericidal, hydrogen peroxide activity being slightly less effective (Meneghini, 1988). In the presence of iron and iron-carrying proteins, H_2O_2 reactions are enhanced, indicating the importance of this element in these type of reactions (Hyslop et al, 1988).

Macrophages can be primed by mediators such as interferon-gamma released by other cells which have the effect of making the cell more susceptible to further activation (Russell, 1986). Stimuli such as IgG immune complexes and phorbol ester (PMA) can induce superoxide and hydrogen peroxide production (Hagenlocker et al, 1990). Other activators of oxygen metabolites include lectins, arachidonic acid and its metabolites, FMLP and opsonised particles (Yasaka et al, 1982).

1.3.10.2. Proteinases.

The proteolytic potential of alveolar macrophages is central to its function. Proteinases have evolved to digest bacteria phagocytosed by macrophages, and for maintenance and turnover of normal tissue. Lysozyme, which is an enzyme secreted in large quantities by activated macrophages, is considered to play a major role in the maintenance of a sterile environment in the lung (Singh et al, 1988). This enzyme can be released after stimulation, either phagocytic or non-phagocytic, and increased levels can be detected in macrophages obtained from smokers (Singh et al, 1988). Other proteinases are presumed to be involved in the digestion of bacteria. The enzyme elastase is another proteinase which is secreted by macrophages (Janoff, 1985) and which can damage elements of the alveolar septum. In addition to these enzymes, collagenases and gelatinases are released by macrophages and these may contribute to damage seen in lung diseases such as emphysema (Werb, 1982).

It is important for normal lung function that activity produced as a result of release of these enzymes is balanced by release of proteinase inhibitors, which inactivate excess enzyme and so normally protect the lung from damage (Janoff, 1985). Antiproteinases secreted by macrophages include Alpha 2-Macroglobulin, which binds the majority of proteinases (Janoff and Carp, 1983), and alpha-1-proteinase inhibitor (Alpha-1-P.I.), which is a major inhibitor of neutrophil elastase (Janoff, 1985). The specific type of antiproteinase secreted by macrophages is likely to depend on environmental factors at the site of inflammation.

1.3.10.3. Cytokines.

The cytokines are a diverse group of mediators released by activated macrophages, although they are also produced by other cells (Warren, 1990; Hogquist et al, 1991). These polypeptide molecules include interleukin-1 (IL-1) alpha and beta, and tumor necrosis factor (TNF) alpha and beta. These two molecules have overlapping functions which include the recruitment and activation of other cells involved in the inflammatory response (Dinarello, 1984; Kelley, 1990).

Interleukin-1.

Interleukin-1 causes acute-phase responses which include fever, changes in circulating leukocyte number and metal concentrations in the blood, as well as changes in serum proteins (Ohkawara et al, 1989). IL-1 is a multifunctional molecule which also causes activation of inflammatory and immune cells (Dinarello, 1984; Larrick and Kunkel, 1988; Dinarello, 1989). A major role of IL-1 in inflammation is the recruitment and activation of neutrophils (Cybulsky et al, 1986; Cybulsky et al, 1989). Neutrophils are mobilized from the marginated pool by emigration through endothelial cell junctions. This process depends on the ability of the leukocytes to adhere to the surface of the endothelium and to respond to certain chemotactic stimuli (Larsen et al, 1990). Evidence shows that IL-1 produces up-regulation of adhesive molecules on the surface of endothelial cells and leukocytes (Dunn and Fleming, 1984; Dustin et al, 1986). These adhesion molecules are also important in both cell-cell and cell-connective tissue interactions.

The production of IL-1 by macrophages is also pivotal for immune responses through the T-cell activating properties of IL-1 (Furie and McHugh, 1989). Macrophages release IL-1 which causes T-lymphocytes to produce IL-2 and up-regulate the

number of IL-2 receptors on the cell surface (Kuhweide et al, 1990). IL-1 thus acts to amplify the immune response by clonal expansion of the T-helper cell population, and by proliferation and activation of B-cells (O'Garra, 1989) leading to antibody production.

Tumor Necrosis Factor.

Tumor Necrosis Factor (TNF) is a cytokine with broadly similar functions to IL-1. The cytokine includes two functionally related proteins, TNF- α , secreted mainly by monocytes/macrophages, and TNF- β or lymphotoxin, secreted by lymphoid cells (Vilcek and Lee, 1991). Both forms of TNF bind to the same cell surface receptors and are similar in their activities. TNF can induce secretion of acute phase proteins, activate neutrophils and up-regulate surface adhesion molecules, especially the CD18 antigen complex (Warren, 1990; Schollmeier, 1990; Walsh et al, 1991). The latter is required for maximal adherence of cells and CR3 (C3bi receptor) function (Gamble et al, 1985). TNF also up-regulates the adhesion molecules on endothelial cells, promoting extravasation of leukocytes (Pichyangkul et al, 1988; Santala and Heino, 1991). Activation of neutrophils by TNF results in degranulation and the production of the respiratory burst, which has important microbicidal functions (Klebanoff et al, 1986). In addition, this cytokine is important in the trafficking of inflammatory cells to the site of inflammation (Ming et al, 1987; Faccioli et al, 1990).

1.3.10.4. Arachidonic Acid and Metabolites.

Activation of alveolar macrophages by a variety of compounds results in the production of bioactive lipids which are derived from metabolites of arachidonic acid (Garcia et al, 1987). Arachidonic acid is produced from cell membrane phospholipids by the action of phospholipase A₂, and can be converted to cyclooxygenase or lipoxygenase products called eicosanoids (Henderson, 1987). The production of prostaglandins and thromboxanes occurs by way of the cyclooxygenase pathway (Henderson, 1987).

Prostaglandin G₂ (PGG₂) is produced by a bis-dioxygenation reaction with arachidonic acid. This first product is unstable and is rapidly converted to PGH₂ and eventually to PGD₂ which is a potent bronchoconstrictor, and PGE₂ an important bronchodilator. Another major bronchoconstrictor produced is PGF₂ α (Hamberg and Samuelson, 1974). The thromboxanes are potent platelet aggregators

(TxA₂), and demonstrate chemotactic activity for leukocytes (Hamberg and Samuelson, 1974).

Leukotrienes are produced by the lipoxygenase pathway. These products are important mediators, which produce effects on airways by constriction, blood vessel permeability, and chemotaxis of PMN (Garcia et al, 1987; Schönfeld et al, 1988). In addition to arachidonic acid metabolites, macrophages and endothelial cells produce Platelet-Activating Factor (PAF), another mediator with overlapping and wide-ranging effects (Zimmerman et al, 1987). This substance in itself has the ability to release arachidonic acid and its metabolites from PMN, thereby enhancing an already present inflammation.

1.3.10.5. Growth Factors.

Macrophages can produce a wide range of factors and compounds which have the ability to induce proliferation in various cell types (Takemura and Werb, 1984). One of the most important of the target cells for lung disease is the fibroblast (Haschek and Witschi, 1979; Reiser and Last, 1981). Fibroblast growth factor produced by macrophages has the ability to produce fibroblast proliferation which may result in increased synthesis of collagen leading to fibrotic lung disease (Keogh and Crystal, 1981). Fibronectin, which is implicated in cell surface interactions and found in extracellular matrix, is secreted by macrophages and is another factor which promotes fibroblast growth (Rennard et al, 1981).

Platelet-derived growth factor (PDGF) is a multifunctional molecule found in serum and which is also secreted by alveolar macrophages (Deuel et al, 1985). PDGF promotes growth of fibroblasts (Bauer et al, 1985) and its local release in the alveolar region has implicated it in the pathogenesis of fibrosis caused by asbestos (Bonner and Brody, 1991).

Another multifunctional molecule involved in inflammation, growth and repair is Transforming growth factor beta (TGF-β) (Wahl et al, 1989). This molecule has similar functions to PDGF causing stimulation of fibroblasts and so may be implicated in fibrosis.

1.4. POLYMORPHONUCLEAR LEUKOCYTES.

1.4.1. Neutrophils in Normal BAL.

The polymorphonuclear granulocytic leukocytes are a family of cells which are short-lived compared with the macrophages and monocytes, and which are produced in the bone marrow (Baggiolini and Dewald, 1985). They have lobulated nuclei and are further classified into neutrophils, eosinophils, mast cells and basophils according to their staining reaction of granules contained in the cytoplasm. The main roles of polymorphs include phagocytosis, release of enzymes and mediators in various host defence responses (Ricevati and Mazzone, 1989; Weiss, 1989).

Polymorphonuclear neutrophilic leukocytes (PMN) comprise over 90% of the circulating granulocyte population, and are smaller cells than macrophages, being approximately 10-20µm in diameter (Goetzl, 1981; Cohen and Rossi, 1983). Neutrophils contain two main types of granules, known as primary and secondary (or specific) granules (Goetzl, 1981). The former contain acid hydrolases, myeloperoxidase and myeloperoxidase which are important enzymes in the killing of bacteria and production of oxygen radicals (Wright, 1982). The latter contain lactoferrin, an iron binding protein, and lysozyme (Baggiolini and Dewald, 1985). Ingested bacteria and microorganisms are phagocytosed by these cells into a phagosome, which fuses with the lysosomes. In the normal lung, there are very few neutrophils in the airspaces (Cohen and Rossi, 1983). There is, however, a large pool of these cells in the pulmonary vasculature, margined on the surface of the blood vessel endothelium (Doerschuk et al, 1987).

When macrophages become activated, PMN chemotaxins are generated, which attract PMN up a concentration gradient between the endothelial cells that line the blood vessels, and into the air spaces (Goetzl, 1981). Recruitment to a site of inflammation by products from activated macrophages can further activate PMN, which leads to enhancement of the initial inflammatory response and often to tissue injury and the development of pathological change (Weiss, 1989).

1.4.2. Eosinophils, Basophils and Mast Cells.

Eosinophils comprise only 2-5% of the circulating granulocytes in normal, non-allergic subjects. These cells are also capable of phagocytosing and killing microorganisms, and like the neutrophils contain granules which can be released on appropriate stimulation (Hallgren et al, 1987; Yukawa, 1990). During degranulation, the contents are released outside the cell to kill targets which are too large to be phagocytosed. The specialized role of the eosinophil is thought to be

related to the immunity to parasitic, particularly helminth infections. The cell can also release histamine, which is important in allergic reactions.

Basophils and Mast cells are found in very small numbers in the circulation, about 0.2% of circulating granulocytes, and the two types are often indistinguishable from one another (Warren and Holford-Strevens, 1986). The mast cell is found mainly associated with mucosal epithelium, and like the basophil has granules which contain heparin, and play a role in allergy and immunity against parasites. Granulocytes other than neutrophils are not of direct interest to the present study and will not be further discussed.

1.4.3. Neutrophil Secretory Products.

As with the macrophage secretion, these are important to normal lung defence but have the capability of mediating lung injury and disease if they are present in sufficient amounts to overcome the defences of the lung.

1.4.3.1. Oxygen Metabolites.

Products secreted by PMN are, by and large, similar to those produced by macrophages and already described above. Of these products, one of the most important is superoxide anion produced by virtue of the high levels of myeloperoxidase present in the neutrophils (Fantone and Ward, 1985). The actual amount of different oxygen metabolites released by stimulated PMN depends on the trigger (English et al, 1981), and on the substratum to which the cells are attached (Nathan, 1987).

1.4.3.2. Proteinases.

The azurophilic granules of the PMN contains the majority of enzymes (Fantone and Ward, 1985). Among the neutral serine proteinases, elastase is by far the most abundant, and has been the most studied because of its likely role in emphysema (Janoff et al, 1977; Janoff, 1985). This enzyme is the only PMN serine proteinase capable of digesting elastin, however other substrates such as the collagens and fibronectin can also be degraded by elastase (McDonald and Kelley, 1980). Regulation of the inflammatory response by elastase as well as other proteinases can be brought about by the activation and inactivation of the complement components C3 and C5a (Orr et al, 1979). The release of PMN enzymes varies

according to the type and intensity of the stimulus, and this is very likely crucial in determining the intensity of the inflammatory response (Perussia et al, 1987).

1.4.3.3. Mediators and Cytokines.

Like the macrophages, PMN can generate inflammatory mediators, although to a lesser extent than is found in macrophages. Arachidonic acid metabolites can be produced, through the cyclooxygenase and lipoxygenase pathways, leukotriene B₄ being the most important product (Weller et al, 1983). This mediator can also act on PMN, inducing chemotaxis, aggregation and adherence to endothelial cells (Vandenbrouke-Grauls et al, 1987). Other mediators produced via the lipoxygenase pathway include Hydroxyeicosatetraenoic acid (HETES) which can produce pro-inflammatory changes in blood vessels and airways (Henderson, 1987).

Secretion of platelet-activating factor (PAF) by PMN can occur after stimulation by various agents (Henson, 1981), and has wide ranging effects on platelets, mononuclear phagocytes, lymphocytes, eosinophils, PMN and endothelial cells (Henson, 1981). Compared with the macrophages, far less is known about the cytokines secreted by PMN. Conflicting studies showed that IL-1 was secreted by PMN in one study (Kusaka and Donaldson, 1990), but could not be detected in another (Hanson et al, 1980).

1.5. THE EPITHELIUM FOLLOWING DUST DEPOSITION.

1.5.1. Type 1 and Type 2 Epithelial Cells.

In the normal lung, over forty different cell types have been described (Crapo et al, 1982). These can be divided into those which provide a defence screen to the lung, for example the macrophages, lymphocytes and neutrophils, and the cells which maintain the structure of the lung (Penney, 1988; Sibille and Reynolds, 1990). Of these structural cells, the types 1 and 2 alveolar epithelial cells are vital in gas exchange, as well as providing a regulatory role in immune reactions (Baughman, 1987; Wilsher et al, 1988).

Type 2 cells can be identified by their cuboidal structure and lamellar bodies which are cytoplasmic inclusions having important secretory and storage functions (Chretien et al, 1985). The cells are found in depressions in the alveolar epithelium, especially in the "corners" of the alveoli (Crapo et al, 1982). In contrast, the type 1 epithelial cell is an elongated flattened cell, covering the surface

of the alveolus, through which gas exchange occurs (Rosenbaum and Picciano, 1978; Weller and Karnovsky, 1986).

A major role for the type 2 cell is that it is a precursor for the type 1 cell (Dobbs et al, 1985). After injury to type 1 cells, the type 2 cells proliferate, gradually flattening, elongating and replacing the damaged cell (Evans et al, 1973; Shami et al, 1986). It is not currently possible to isolate type 1 cells from lung tissue and studies carried out on alveolar epithelial cells have focussed on the type 2 cells. Other studies have utilized type 2 alveolar epithelial cells lines (Donaldson et al, 1992a).

1.5.2. Secretory Capacity of Epithelial Cells.

Type 2 cells synthesize and secrete surfactant, a complex mixture of saturated phosphatidylcholine, cholesterol, phosphatidylglycerol and unsaturated lipids (Rooney, 1985). The surfactant is important during respiration where it plays a role in preventing the alveoli from collapsing during expiration when the pressure of air is reduced (Shannon et al, 1987). Surfactant secretion and composition is regulated by hormones, neurotransmitters and growth factors (Rooney, 1985), and receptors for hormones have been identified on the surface of isolated cells (Pysher et al, 1977). In addition to their secretory function, type 2 cells have alkaline phosphatase activity which may be detected on the cell membrane and is a marker of cell differentiation (Richards et al, 1987; Edelson et al, 1988).

Complement components C3 and C5 have been shown to be secreted by type 2 cells (Strunk et al, 1988; Rothman et al, 1989b). This has implications for lung injury when dust depositing in the lung, especially organic dusts which may be contaminated with bacterial endotoxin, can generate chemotaxins for inflammatory cells from complement components C3 and C5 (Olenchock, 1978; Donaldson et al, 1990).

1.5.3. Epithelial Injury.

A major role played by the alveolar epithelium is in the maintenance of a permeability barrier, important in regulating cellular and fluid traffic from the blood, through the interstitium and into the alveolar spaces and in the opposite

direction (Rosenbaum and Picciano, 1978). The epithelial barrier may become compromised as a result of inflammatory processes, due to the inhalation of toxic particles and gasses present in environmental substances such as cigarette smoke (Hunninghake and Crystal, 1983; Panus et al, 1989). Toxic effects may be a direct result of interactions between inhaled toxic materials and the epithelium. They may also be brought about by release of toxic material from inflammatory cells attracted to damaged lung (Simon et al, 1986; Holt, 1987; Donaldson et al, 1992a). In other cases, where lymphocytes are present in increased numbers as a result of immunological stimuli in workers exposed to organic dusts (Keller et al, 1984), type 2 epithelial cell and immune cell interactions may take place, resulting in epithelial injury (Donaldson et al, 1990).

As mentioned previously, replacement of damaged alveolar epithelium occurs via the proliferation and differentiation of type 2 cells. In conditions such as fibrotic lung disease and silicosis, there is marked hyperplasia of type 2 cells (Kawanami et al, 1982), and in the latter disease, accompanying alveolar proteinosis (Heppleston, 1975). This condition may exert additional regulatory effects on the lung through the excessive amounts of surfactant which is produced by the hypersecretory type 2 cells (Schuyler et al, 1980; Kawada et al, 1989). In some cases, the accumulation of surfactant may be attributed to decreased numbers of macrophages which are a main route for surfactant removal (Miles et al, 1988).

1.6. ADHESION MOLECULES.

1.6.1. Structure.

Adhesion between cells and cells, and between cells and the extracellular matrix are both important interactions necessary to maintain the normal structure of the lung. Another important role of cell adhesion is in the attachment of circulatory leukocytes to endothelial cells prior to migration from the bloodstream to sites of inflammation. Molecules which mediate these events have been characterized on the surface of cells such as lymphocytes, macrophages and neutrophils, and also on endothelial cells of blood vessels and capillaries (Kishimoto et al, 1989).

The interactions between cells is mediated largely by a group of adhesion molecules known as integrins. One of the best studied and important molecular complexes is CD11/CD18. This is a membrane bound glycoprotein, comprised of three distinct subunits CD11a b and c (Aranout, 1990); Alternatively, these receptors are known as LFA-1 (Lymphocyte Function Associated antigen-1), CR3 and p150,95

corresponding to CD11a, CD11b and CD11c respectively. These subunits are non-covalently linked with a common beta sub-unit (CD18), to form a complex which spans the cell membrane, and which is stabilized by calcium and magnesium cations (Patarroyo, 1989; Dransfield, 1990). These ions are important in adhesive interactions between other adhesion molecules and their counter receptor or ligand. The ligand for LFA-1a is ICAM-1 (Intercellular Adhesion Molecule-1, or CD54) (Diamond et al, 1990). This is an inducible antigen, expressed on the surface of leukocytes, endothelial cells and epithelial cells, after stimulation. Molecules such as bacterial lipopolysaccharide (LPS or Endotoxin), or the pro-inflammatory cytokines IL-1 and TNF are potent activators of adhesion molecules (Webb et al, 1991).

The CR3 receptor (CD11b) is a ligand for iC3b, the cleavage product of C3 and this ligand is also the counter-receptor for p150,95 (CD11c). Cell surface adhesion molecules have a widespread distribution, and are present in varying amounts on different cell types. The CD11a/CD18 complex is found on all leukocytes, CD11b and c/CD18 on monocytes, macrophages and neutrophils (Montefort and Holgate, 1991).

1.6.2. Function.

Various leukocyte functions have a mandatory requirement for binding via the CD11/CD18 complex. In the case of T lymphocytes, mitogen and antigen-driven proliferation, and T-cell-mediated cytotoxicity are all dependent on CD11a/CD18; in the case of B lymphocytes, aggregation and immunoglobulin production rely on the same heterodimer (Patarroyo, 1989). The granulocytes, monocytes and NK cells express all three CD11/CD18 heterodimers, no doubt highlighting the interactions between the cells and cells of the lymphoid series. The adhesion between T and B lymphocytes to activated endothelial cell monolayers is a CD11a/CD18 dependent process, and also relies on the presence of divalent cations (Aranout, 1990).

In granulocytes and monocytes the CD11/CD18 complex is critical for adhesion between cells and the endothelium (Kishimoto et al, 1989), and it also plays an important role in the phagocytosis of opsonised particles with neutrophils, and possibly macrophages (Montefort and Holgate, 1991). Another crucial role is in chemotaxis where cells must migrate from the blood stream and into the tissues to sites of inflammation.

1.6.3. Activation and Regulation.

Activation of integrin molecules can occur after the cross-linking of other cell-surface molecules, in particular CD14 (Lauener et al, 1990; Schumann, 1992), the receptor for lipopolysaccharide and lipopolysaccharide binding protein. This important event occurs in infection when LPS becomes bound to LPS binding protein (LBP). Lipopolysaccharide binding protein and other acute phase proteins are secreted in large amounts by the liver after stimulation with LPS. Binding of the LPS/LBP complex to CD14 can produce activation of the CD11/CD18 complex, and result in the homotypic aggregation of monocytes (Lauener et al, 1990; Schumann, 1992). Activation is an energy requiring process which is regulated by ATPase and is inhibited at 4°C (Patarroyo and Jondal, 1985). The CD14 receptor is involved in other intracellular signalling events through the action of the second messenger Protein Kinase C (Lauener et al, 1990).

The role of cytokines in the regulation and activation of leukocyte adhesion molecules is well established. In endothelial cells, treatment with interferon-gamma, TNF, or IL-1 increases the adherence of normal unstimulated lymphocytes (Patarroyo, 1989). Other molecules present at sites of inflammation, including leukotriene B₄, and Platelet-Activating Factor, (PAF) enhance the adhesion of neutrophils to endothelial cells (Patarroyo and Makgoba, 1989); this property is shared by other classical leukocyte activating agents such as LPS (Feist et al, 1989). In unstimulated granulocytes and monocytes, intracellular pools of CD11b/CD18 and CD11c/CD18 are present which can be translocated to the cell membrane very quickly after stimulation by FMLP and PMA leading to the functional effects mentioned above (Lauener et al, 1990).

1.6.4. Role in Aggregate Formation.

During an inflammatory response, many different mediators are released from inflammatory cells at the site of inflammation. Some of these molecules especially TNF and IL-1, regulate the expression of integrins (Santala and Heino, 1991). In addition, in the same study, IL-1 β and TGF- β (Transforming Growth Factor) were shown to act in a synergistic manner in potentiating integrin activation. Homotypic aggregation is a phenomenon associated with monocyte activation (Lauener et al, 1990). Aggregation results from increased expression or up-regulation of surface adhesion molecules. Monoclonal antibodies directed against the CD11a, CD18 and ICAM-1 receptors (Tamatani et al, 1991) have shown that these receptors are responsible for aggregation of activated T-lymphocytes, and in adherence of leukocytes to activated endothelium during inflammation.

1.7. AIMS AND SCOPE OF THE PRESENT STUDY.

The Mechanisms of Organic Dust-mediated Inflammation and Leukocyte Activation in the Rat Lung.

The previous two wool dust studies identified potential detrimental effects in the lung function of workers in wool textile mills, and related the findings to the airborne mass concentration of dust. A major conclusion was that the dust caused bronchitis. The present study was undertaken to examine some of the interactions between wool mill dust and the lung using a rat model. Dust was instilled intratracheally and the subsequent responses of the rat lung were examined. The study investigated several different aspects of lung inflammation and immunity, and the interactions between the cells and mediators in an attempt to understand how bronchitis might arise in the lungs of wool dust-exposed workers. Because grain dust is another organic dust causing symptoms of bronchitis and is also part of an HSE study, it was also included.

1.7.1. Organic Dust-mediated Inflammation.

One of the main hypotheses tested in this study was that organic dust depositing in the lung gives rise to inflammation which may underlie the symptoms of bronchitis. Since only very small amounts of dust were available, dust was instilled intratracheally as a single bolus. Cells which are newly recruited to the alveolar space as a result of dust exposure can be assessed by broncho-alveolar lavage. Increases in total cell number, as well as the cell types making up the lavageable population can be an important indicator of the extent of inflammation caused by dust. Cells which are lavaged from the lung after dust exposure can also be used in functional studies to assess mechanisms of inflammation.

1.7.2. Epithelial Injury and Cytotoxicity.

Dust which penetrates to the respiratory zone of the lung, encounters alveolar epithelial cells and phagocytic macrophages. Potentially, dust may cause direct injury to both these cell types, by damaging the cell membrane resulting in lysis of the cell, or detachment from the lung surface. Denudation of the basement membrane could cause initiation of an inflammatory response, which may become chronic and

result in pathological changes within the lung. Injured cells and fragments of lysed cells could themselves produce inflammation because they are chemotactic for leukocytes. Accumulation and activation of macrophages and neutrophils at sites of inflammation could result in the bronchitic symptoms described in organic dust-exposed workers.

1.7.3. Secretion of Pro-inflammatory Mediators.

In the absence of any direct toxic effect of dust on the cells of the lung, the phagocytosis of wool dust may be sufficient to activate alveolar macrophages. These macrophages may, in response to the dust secrete molecules which could enhance inflammation, including oxygen radical, proteinase, and cytokines. One important cytokine mediator of inflammation is tumor necrosis factor (TNF), which is produced in large amounts on stimulation with LPS. Tumor necrosis factor released locally in the airspaces enhances inflammation by producing stimulation of resident cells, and upregulation of adhesion molecules on endothelial cells.

1.7.4. Role of LPS in Causing Local Activation of the Immune System.

An organic dust such as wool dust is potentially antigenic when inhaled into the lung. Other factors in or on the dust may also play a role in inflammation and immunity. Endotoxin is such a substance being ubiquitous in nature due to its production by gram negative bacteria, which are present in most, if not all, organic dusts.

In the upper airways, dust depositing on the muco-cilliary escalator is carried upwards for eventual clearance from the lung. The other main clearance pathway is via the alveolar macrophages, which phagocytose dust deposited below the level of the muco-cilliary escalator (MCE) and the terminal bronchioles and can translocate the particles to the lymph nodes, although more may be cleared by macrophages using the MCE. Macrophages which are stimulated and translocate to the lymph nodes could release cytokines producing direct effects on lymphocytes. In addition, endotoxin on the wool dust could cause direct activation of the lymph node lymphocytes if translocated there by the alveolar macrophages.

The combined effects of cytokines, endotoxin or other components associated with wool dust could be stimulation of lymphocytes. By culturing mediastinal lymph node lymphocytes from animals treated intratracheally with wool/grain dusts with lymphocyte mitogens, the degree of activation or priming may be assessed by measuring uptake of tritiated thymidine.

1.7.5. Adhesion Molecule Activation.

Adhesion molecules are important in cell/cell and cell/matrix interactions. Although these molecules play a key role in normal circumstances, during inflammation they are up-regulated by cytokines, as well as various other molecules, and are crucial for the migration and chemotaxis of inflammatory cells. Adhesion molecules can be demonstrated on cells and tissues by using monoclonal antibodies directed against these molecules.

1.7.6. Histology.

Pathological changes can be identified by examining the lungs of dust exposed rats. A sequence of lungs exposed to dust for varying time points can allow the progress of pathological change to be monitored.

1.7.7. Summary.

The aim of the study was to determine the mechanisms by which organic dust could cause symptoms of bronchitis in dust-exposed workers. The mechanisms were hypothesized and tested.

1. Organic dust causes a direct toxic effect towards epithelial cells and alveolar macrophages.
2. Organic dust causes increased secretion of pro-inflammatory cytokines by alveolar macrophages.
3. Organic dust causes up-regulation of adhesion molecules on alveolar macrophages.
4. Organic dust causes stimulatory effects in the lung lymph nodes.

MATERIALS AND METHODS.

MATERIALS AND METHODS.

2.1. DUST PREPARATION.

2.1.1. Dust Collection.

Dusts were collected from two wool processing mills in the North of England. Dusts were designated S (start) and M (middle) which represented opening /blending and combing processes respectively. A series of six IOM static inspirable dust samplers (Mark et al, 1985) were placed at each site in the dustiest zones. Samplers were operated for a full work shift and the dust collected on Gelman GLA filters with a 5 μ m pore size (Gelman Hawksley, Northampton). Dust was removed from filters with a soft brush. Dust from each mill was pooled into a tube, weighed and mechanically rotated for 24 hours to ensure mixing and these samples were stored at -20°C until required.

Samples of grain dust were collected from the ledges of a barn which stored wheat and barley. The dust was sieved through a 200 μ m mesh, followed by a 45 μ m mesh, by shaking mechanically for 30 minutes. The dust so obtained was less than 45 μ m diameter, and this was used in all subsequent assays.

2.1.2. Preparation of Dust Leachates.

Samples of leachate were prepared by mixing ledge wool dust (obtained from wool mills by sweeping ledges and surfaces) and sieved grain dust in PBS at a concentration of 5mg/ml at room temperature for 24 hours. Solutions were then spun at 3000 rpm for 15 minutes to remove large fragments and then finally filtered through 0.22 μ m filters to sterilize the leachates. A separate group of dust leachates was prepared in F-10 medium (Gibco, Paisley) containing 0.2% Bovine Serum Albumin (BSA), (Flow Labs., High Wycombe) for use in the TNF assay (section 2.9.2). This medium contained negligible amounts of endotoxin (LPS) (<1 ng/ml), as defined by the manufacturers assay.

2.1.3 Washed Wool Dust and Their Leachates for Intratracheal Instillation.

Wool dust was resuspended at a concentration of 2mg/ml in sterile saline and rotated on a rotary mixer at room temperature for 5 minutes, 2 hours and overnight. After

each time point, the tubes were spun at 3000 rpm for 15 minutes, after which the leachate was removed and the dust resuspended at 2mg/ml in a fresh aliquot of sterile saline. One milligram of the dust in a volume of 0.5ml was instilled into rat lungs for 1 day. Leachates were also instilled into a separate group of animals, 0.5ml/animal, which represented the amount of material leached from 1mg of dust.

2.1.4. Endotoxin Estimations of Leachates.

The "Coatest" (ICN, Flow, High Wycombe, Bucks) Limulus Amoebocyte Lysis (LAL) based spectrophotometric assay was used to assess the endotoxin content of the wool/grain leachates according to the manufacturers instructions.

All the reagents used in the wool study were examined for their LPS content and were found to be negligible (<1 ng/ml).

2.1.5. Depletion of Endotoxin using Polymyxin.

Leachates prepared from grain and wool dusts were depleted of endotoxin by passage down columns of polymyxin immobilized onto a solid phase substratum (Pierce, Chester, England) according to the manufacturers instructions. Each leachate and control medium were passed twice and the total endotoxin content estimated before and after depletion (Table 2).

LEACHATE	ENDOTOXIN (ng/ml)	
	PRE COLUMN	POST COLUMN
WOOL S	21.99	0.14
WOOL M	19.76	0.16
GRAIN	14.99	0.14
LPS	22.45	0.13

Table 2. The endotoxin content of dust leachates before and after polymyxin treatment.

2.2. CHARACTERISATION OF WOOL DUST.

2.2.1. Scanning E.M. (SEM).

Wool dust was resuspended at a concentration of 1mg/ml in sterile PBS. The dust suspension was filtered onto 5µm nucleopore filters (Costar U.K., High Wycombe, Bucks) which were allowed to dry, mounted on aluminium SEM stubs (Agar Scientific, Essex, England) and gold coated. Samples were viewed in a Hitachi scanning electron microscope.

2.3. IN VIVO TECHNIQUES.

2.3.1. Rats.

Male adult Han rats supplied by Charles River U.K., Margate, Kent, were used throughout.

2.3.2. Intratracheal Instillations.

Groups of 3 rats were anaesthetized with an intraperitoneal injection of 0.1ml Valium (Roche, Welwyn Garden City) at a concentration of 5mg/ml, followed by an intramuscular injection of 0.05ml Hypnorm (Jansen Pharmaceuticals) containing 10mg/ml Fluanisone and 0.315mg/ml Fentanyl citrate. An incision was made to expose the trachea and 0.5ml of wool dust suspension contained in PBS (Gibco, Paisley) at a concentration of 2mg/ml were instilled via a blunted needle. The incision was closed using surgical clips. The animals were further injected intramuscularly with 0.2ml of Naloxone (Sigma, Poole, Dorset) contained in PBS at a concentration of 0.3mg/ml, to reverse the effects of anaesthesia. Animals were conscious within a few minutes and showed no ill effects.

2.3.3. Broncho-alveolar Lavage (BAL).

At selected time points, animals were sacrificed with a single intraperitoneal injection of Nembutal, the thoracic cavity opened and the lungs cannulated and removed. The lungs were then sequentially lavaged with four 8ml aliquots of saline at 37°C and pooled into a single tube. Cells were spun at 1000 rpm in a refrigerated centrifuge for 10 minutes, and resuspended in F-10 medium (Gibco, Paisley) containing 2% BSA (Fraction V, Sigma, Poole, Dorset). Total cell counts

and viability were measured, and cytocentrifuge smears prepared which were stained with Diffquick (Merz Dade, Switzerland). One hundred cells/cytospin were counted to obtain differential cell counts in the different treatments. Cytospins for immunostaining were prepared, fixed in acetone for 10 minutes, and stored at -20°C until required.

2.4. IN VITRO TECHNIQUES.

2.4.1. Aggregate Characterization.

Characterization of the cell aggregates from BAL of rats exposed to wool dust was carried out using mouse anti-rat monoclonal antibodies (Serotec, Oxford) directed against surface markers on macrophages (Serotec MCA 342; ED2 clone), and T-lymphocytes (Pan T, Serotec, MCA 52; OX 19). Normal goat serum (NGS; Scottish Antibody Production Unit, SAPU, Carlisle) diluted to 5% with tris-buffered saline (TBS) was used as a blocking agent to prevent non-specific staining followed by the appropriate monoclonal antibody.

The monoclonal antibodies were further diluted in 5% NGS to give optimum staining. All slides were then washed thoroughly in TBS for 10 minutes with two changes of solution. Subsequent steps involved the use of a commercially available alkaline phosphatase staining kit (ICN Immunochemicals, High Wycombe, Bucks) to visualise the macrophage/T-lymphocyte aggregates. When the desired degree of staining was achieved, slides were washed in TBS and counter-stained in Harris haematoxylin for 30 seconds, rinsed in tap water and mounted in PBS/Glycerol (1:1).

In addition to the test slides, controls consisting of lymph node and one set omitting the primary antibody were included. Cells were evaluated by counting 10 separate aggregates from each of the test conditions in triplicate. Within each aggregate, numbers of positive, negative and polymorphonuclear neutrophils (PMN) cells were noted, as were the total number of cells making up each aggregate. Grain treated BAL was not assessed for aggregate characterization due to a delay in receiving the grain dust (criteria for aggregate identification is given in section 2.5.2).

2.5. AGGREGATION ASSAY.

2.5.1. Comparison of Assay Systems.

Rat alveolar macrophages were harvested from control lungs and suspended in each wool leachate prepared in F-10 + 0.2% BSA, depleted wool leachate, macrophage supernatant (section 2.9.2), TNF (National Standards Board, Potters Bar, Herts.), phorbol myristate acetate (PMA), LPS or fibronectin (Sigma, Poole, Dorset) at a concentration of 2.5×10^5 cells in 200 μ l of solution. Standard TNF was diluted to 100 units/ml in F-10 + 0.2% BSA; PMA and LPS were diluted to 10 μ g/ml and fibronectin to 1 μ g/ml in F-10 + 0.2% BSA. Cells were also suspended in wool and grain dusts at a concentration of 100 μ g/ml.

Three different assay systems were compared, all of which yielded similar size and number of aggregates. Cells were incubated at 37°C for 24 hours, in teflon coated wells, 24-well plates or Tissue-Tek slides (Miles laboratories, Illinois) and the non-adherent aggregates collected and cytocentrifuge preparations made which were then stained with Diffquik. The number of cells present in each of 10 randomly selected aggregates per cytospin were counted.

2.5.2. Definition of an Aggregate.

An aggregate was defined as a cluster of 10 or more contiguous cells which remained after gentle pipetting and cytospin preparation. With reference to Plate 1, there are 11 aggregates which are clearly distinguishable from groups of cells that are merely single cells that are touching. The reproducibility of the counting procedure was verified with different observers counting the same slide, and with one observer reading the same slide twice and results obtained were very closely similar in each case.

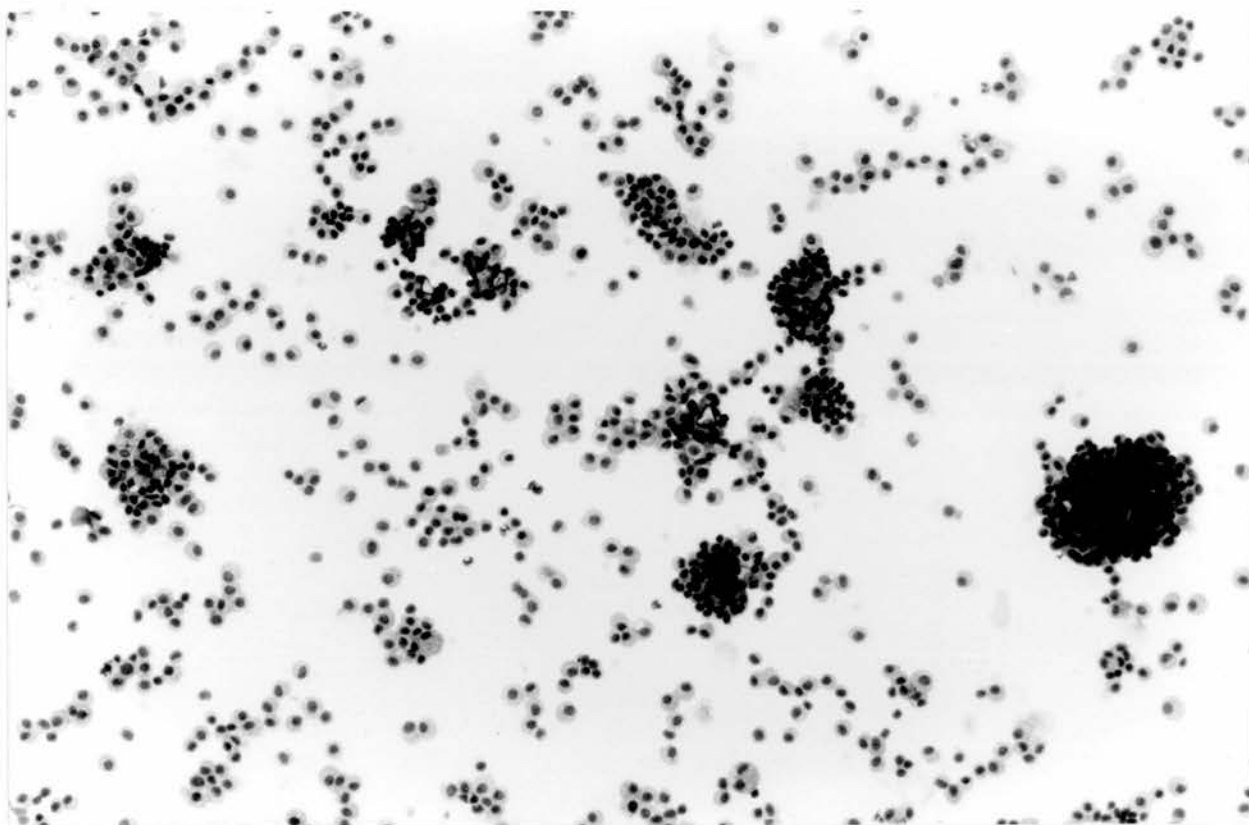


PLATE 1. The typical appearance of aggregates
Original Magnification
x 200.

2.5.3. Staurosporine.

Staurosporine (Sigma, Poole, Dorset) was included with PMA, LPS and whole fractions of wool and grain dusts as described above, at a concentration of $2\mu\text{M}$.

2.5.4. Removal of Calcium Ions.

In some experiments, assay medium was depleted of calcium and magnesium ions using potassium EDTA (BDH, Paisley) at a concentration of 2.5mg/ml .

2.6. ADHESION MOLECULES.

2.6.1. Monoclonal Antibody to LFA-1 alpha and beta and ICAM-1.

The hybridoma cells were prepared and kindly supplied by Dr. Takuya Tamatani of the Tokyo Metropolitan Institute of Medical Science. Hybridomas which secreted monoclonal antibodies against LFA-1 alpha and beta (rat CD11a/CD18 respectively), and ICAM-1, the counter receptor for LFA-1, were cultured in RPMI-1640 medium (Gibco, Paisley) containing 10mM Hepes, 2mM L-Glutamine, 1mM sodium pyruvate, 10 μ l of 5M 2-Mercaptoethanol, 100U/ml penicillin, 100 μ g/ml streptomycin and 10% foetal calf serum (FCS) (complete medium). Cells were cultured in 75cm² flasks until the colour of the medium became yellow (about 7 days), at which point the medium was collected, spun to remove cells, and stored at -70°C until required.

2.6.2. Effect of Anti LFA-1 alpha and beta, and Anti ICAM-1 on Aggregate Formation.

Alveolar macrophages were cultured in 24 well plates with either PMA or LPS at the concentrations used previously (section 2.5.1). Hybridoma supernatants at different dilutions, or complete medium which served as a control were added. Since the hybridoma supernatant must necessarily contain the metabolites of the hybridoma cells, we wished to control for any effect that these might have on aggregate formation. We therefore prepared a supernatant from rat lymphocytes cultured for the same duration as the hybridoma cells and referred to as a "lymphocyte supernatant control". An irrelevant antibody Pan-T (Serotec, Oxford) MRC OX-19, was included at the same dilutions as the hybridoma supernatants.

2.6.3. Staining of Adhesion Molecules.

Aggregates of cells lavaged from animals treated with wool and grain dusts were used to prepare cytocentrifuge smears which were fixed in acetone for 10 minutes. Slides were transferred to a PBS/Tween wash solution containing 0.05% tween for 5 minutes, after which all subsequent staining was carried out in a humidified chamber at room temperature. Each slide was flooded with 1% hydrogen peroxide solution for 5 minutes to block endogenous peroxidase activity, and washed again for 5 minutes in PBS/Tween. Slides were then treated for 20 minutes with 5% normal

horse serum diluted with 0.05% Tween in PBS. After this step, slides were not rinsed, but carefully wiped to remove excess serum.

One hundred microlitres of each hybridoma supernatant (see section 2.6.1) were placed on the appropriate slide (4 slides/dust treatment, LFA-1a, LFA-1 β and ICAM-1); one control slide from each treatment received only PBS/Tween solution. An irrelevant antibody (PAN-T, OX 19, Serotec) was included in the staining procedure. Slides were incubated for 1 hour at room temperature, and then washed for 5 minutes in PBS/Tween.

Biotinylated horse anti-mouse IgG (Vector labs, Peterborough) was diluted 1:200 in PBS/Tween solution and added to each slide. Slides were incubated for 20 minutes at room temperature, followed by a 5 minute wash in PBS/Tween. Visualization of the bound antibody was achieved using a commercially available kit comprising an avidin/peroxidase complex and a peroxidase substrate kit (Vector labs; ABC, AEC kits respectively) according to the manufacturers instructions. Slides were counterstained with Harris haematoxylin, washed in tap water, rinsed in PBS/Tween and mounted using PBS/glycerol (1:1).

2.7. CYTOTOXICITY ASSAY.

2.7.1. Macrophage Labelling.

Alveolar macrophages were obtained from rats by BAL as previously described (section 2.3.3). Cells (1.5×10^6) were resuspended in 1ml PBS containing 7.5MBq ^{51}Cr (Amersham, Bucks.) and incubated in a water bath at 37°C for 20 minutes, washed twice with PBS, and resuspended at 2.5×10^5 cells/ml in F-10 medium (Gibco, Paisley) containing 2% BSA (Fraction V; Sigma). Two hundred microlitres of cell suspension were added to Linbro microtitre plate wells (Flow Labs., High Wycombe) and incubated at 37°C in 5% CO₂ for 1 hour. The medium was replaced with 200 μ l of previously prepared dust suspensions contained in F-10 + 2% BSA in triplicate groups, giving final concentrations of 1, 10 and 20 μ g/well. Plates were incubated for 24 hours at 37°C in 5% CO₂. After centrifuging for 5 minutes at 1000 rpm one hundred and fifty microlitres of supernatant were removed from each well and counted in a gamma counter.

2.8. DETACHMENT ASSAY.

2.8.1. Alveolar Epithelial Cell Line.

Cells of the A549 epithelial cell line, derived from a human lung carcinoma (Lieber et al, 1976) were maintained in routine culture in minimal essential medium (MEM) plus 10% heat inactivated foetal calf serum, containing penicillin and streptomycin (Complete Medium) (Gibco, Paisley). These cells retain the main morphological features of alveolar type 2 cells, having prominent lamellar bodies and the ability to secrete surface active material (unpublished observation).

2.8.2. Epithelial Injury Assay.

Lung epithelial cells (A549) were removed from continuous culture with 0.1% trypsin/EDTA solution and resuspended in complete medium at a concentration of 2.5×10^5 cells/ml, containing ^{51}Cr (Amersham, Bucks) at an activity of 370KBq/ml. Two hundred microlitres of the labelled cell suspension were plated into Linbro microtitre plate wells and incubated overnight at 37°C in a humidified atmosphere containing 5% CO₂.

Monolayers were washed twice with PBS and 200µl of the previously prepared dusts, leachates and endotoxin were added to triplicate wells, at the appropriate concentration. Plates were incubated at 37°C in 5% CO₂ for 4 hours. As negative controls, wells which contained medium and cells alone were set up. To obtain total radio-label uptake, cells were exposed to 0.1% Triton-X (Sigma, Poole, Dorset) to produce lysis.

After 4 hours incubation, the amount of ^{51}Cr released from the A549 cells was measured by aspirating 50µl of supernatant from each well and measuring in a gamma counter. The result obtained was multiplied by four to give total counts released and attributable to cell lysis. Cells which were injured and had become detached from the plate were measured by removing the remaining supernatant and washing the wells with 2 x 200µl aliquots of PBS. These fractions were pooled and counted by gamma counter. The 150µl of supernatant containing counts due to cell lysis was subtracted from the total pooled counts to give counts due to detached cells alone.

2.9. TUMOR NECROSIS FACTOR.

2.9.1. L929 cell line.

L929 cells, derived from mouse connective tissue cells, were obtained from the European Collection of Animal Cell Cultures (Porton Down, Wilts.), and cultured according to the suppliers recommendations. Cells were sub-cultured once per week to maintain a continuous supply. The medium, MEM + 10% foetal calf serum was changed twice per week.

2.9.2. Preparation of Alveolar Macrophage Supernatants.

Alveolar macrophages were harvested from control rat lungs by broncho-alveolar lavage and resuspended at a concentration of 1×10^6 cells/ml in F-10 medium + 0.2% BSA. One millilitre of this cell suspension was added to each well of 24-well plates (Linbro, Flow Labs) and incubated at 37°C for 1 hour after which the medium was removed and the adherent cells washed twice with PBS. The medium was replaced with 1ml of wool and grain dust suspension at a concentration of 1mg/ml in F-10 + 0.2% BSA. Plates were incubated at 37°C for 24 hours. Supernatants were harvested and spun at 3000 rpm for 15 minutes, aliquotted and stored at -70°C until required.

Endotoxin from E.coli serotype 011:B4 (Sigma, Poole, Dorset) was made up to 1mg/ml in PBS, and stored at -70°C. Some experiments comprised treating macrophages with LPS at various concentrations. Supernatants from leachate treated macrophages were prepared by treating adherent macrophages with 1ml of leachate, and endotoxin depleted leachate.

2.9.3. Preparation of Macrophage Supernatants from C3H; C3H/HEJ Mice.

Macrophages from C3H/HEJ mice are unresponsive to the effect of LPS (Rosenstreich et al, 1978), and the related C3H mice are normal responders. Peritoneal exudate cells (PEC) were treated with dust leachates (section 2.1.2) to prepare supernatants. These were then assessed for their TNF level, allowing any involvement of endotoxin in the leachates to be estimated.

Mice were treated with a single intraperitoneal injection of 0.5ml of 1% thioglycollate broth. Three days later, mice were killed with ether, and the peritoneal cavity lavaged with 3 x 2ml volumes of PBS containing heparin at a final

concentration of 100 Units/ml. Lavage fluid was centrifuged at 1000 rpm for 5 minutes, resuspended in F-10 + 0.2% BSA and cells counted. Cells were adjusted to 1×10^6 cells/ml and 1 ml added to each well in 24-well plates which were incubated at 37°C for 1 hour. The medium was then removed, adherent cells washed twice with PBS, and dust leachates pre and post-polymyxin treatment (section 2.1.2) added to the wells. Plates were incubated at 37°C overnight, supernatants removed, spun at 3000 rpm for 15 minutes and stored at -70°C until they were assayed.

2.9.4. L929 Culture and TNF Estimations.

L929 cells were removed from continuous culture using 0.1% trypsin/EDTA (Gibco, Paisley), and resuspended in MEM complete medium. Cells were adjusted to 3×10^5 cells/ml in complete medium, and 100µl added to each well in 96-well plates. Plates were incubated at 37°C overnight in a humidified atmosphere with 5% CO₂.

On day 2, the medium in each well was replaced with MEM medium containing 5% FCS, 1µg/ml actinomycin D (Sigma, Poole, Dorset), without antibiotics. The top row of each plate received an additional 50µl of medium containing actinomycin D at a concentration of 2µg/ml. The bottom row of each plate served as the control wells (six wells) and the remaining set of six wells were used as blanks when reading the plates.

The top row of each plate received a final 50µl of test supernatant, each supernatant was measured in triplicate wells. A multichannel pipette was used to double dilute 100µl from the top row of wells down the plate, ending before the bottom control wells, and discarding the final 100µl of medium. One set of wells contained a TNF standard (National Standards Board, Potters Bar, Herts.), at a concentration of 100Units/ml. Plates were then incubated for a further 24 hours at 37°C.

2.9.5. Harvesting and Reading.

On day 3, the supernatant in each well was removed and replaced with 50µl of 1% crystal violet stain in 20% methanol for 2 minutes. Each plate was then washed with tap water and allowed to dry, after which each well received 50µl of 20% acetic acid to solubilize the stained cells. Any bubbles were removed and plates were read at 540nm using an automatic plate reader (Dynatech, Billingshurst, Sussex) interfaced with a personal computer (Amstrad, PC1512DD).

2.9.6. TNF Calculations.

The mean O.D. for each sample dilution was calculated as a percentage of the untreated control. The sample dilutions were converted to log e values and regressions performed using % sample O.D. v log E dilution. The log e dilution value giving an O.D. of 50% of the control cells was calculated using the equation

$$\%O.D. = A + B * \log e \text{ dilution}$$

$$\log e \text{ Dilution (50\%)} = (50-A)/B$$

When 50µl of undiluted supernatant was added to the top row of wells, this resulted in a final concentration of 1/4. This was double diluted down the plate, resulting in final dilutions of 1/8, 1/16, 1/32, 1/64, 1/128. These represented the dilutions which were converted to log e values in the calculations of TNF. The TNF standard was used to calculate the number of units of TNF in the samples by relating them known number of TNF units giving 50% control O.D.

2.10. LYMPH NODE RESPONSES.

2.10.1. Lymph Node Cell Thymidine Uptake.

Lymph nodes were aseptically dissected from the mediastinum of wool and saline-treated rats and immediately placed in RPMI medium containing 20mM Hepes buffer, 100mM L-Glutamine, 2-Mercaptoethanol (Gibco, Paisley) and Penicillin and Streptomycin. Lymph nodes were disaggregated by gently homogenizing through a fine wire mesh and resuspending in RPMI medium supplemented with 10% foetal calf serum (complete medium), (Gibco, Paisley) which had been heat inactivated at 56°C for 30 minutes. Cell suspensions were washed twice in complete medium before resuspending at a concentration of 2×10^6 cells/ml. One hundred microlitres of cell suspension were added to triplicate groups of wells in Linbro microtitre plates (Flow labs., Hertfordshire).

Each group was treated with 100µl of the lectin phytohaemagglutinin (PHA) (Sigma, Poole, Dorset) to give final concentrations of 10 and 50µg/ml. In addition, one triplicate group received medium alone. Plates were incubated for 48 hours at 37°C in a humidified atmosphere with 5% CO₂. Each well was then pulsed with 20µl of 1.85 MBq/ml (37KBq/well) ³H-Thymidine (Amersham, Buckinghamshire) for an additional 24 hours. Cells were harvested using an Ilacon cell harvester and

collected on glass fibre filter papers which were counted using liquid scintillometry (Unisolve E, Fisons, Loughborough) in a Phillips β -counter.

2.10.2. Characterization of Lymph Node Cells.

Lymph node cell suspensions (section 2.10.1) were used to prepare cytocentrifuge smears which were immediately fixed in acetone for 10 minutes. These were wrapped in aluminium foil and stored at -20°C until required. Before the slides were stained, they were immediately placed in cold acetone and brought to room temperature to prevent condensation formation. Slides were placed in PBS containing 0.1% BSA for 10 minutes at room temperature, after which they were treated with 5% normal mouse serum diluted in PBS/BSA to prevent non-specific binding. The following mouse anti-rat monoclonal antibodies (Serotec, Oxford) were used at a dilution of 1:100 in PBS/BSA, and incubated on the cytospins for 1 hour at room temperature. Pan-T cell (MCA 339; OX-52), anti-macrophage (MCA 342; Clone ED2), Helper T-cell CD4 (MCA 153; OX 35 clone), Suppressor T-cell CD8 (MCA 48; OX 19 clone), anti-rat granulocyte (MCA 149A MOM/3F12/F2 clone). A control consisting of only PBS/BSA was included, and the anti-rat granulocyte monoclonal served as an irrelevant antibody. Lymph node cells from rats which had been treated with wool dust M and saline for periods of 1 and 7 days were characterized.

After this treatment, slides were washed in PBS/BSA, two 5 minute changes, and incubated with FITC-labelled sheep anti-mouse IgG (Scottish Antibody Production Unit, SAPU, Carlisle), diluted 1:100 in PBS/BSA for 30 minutes at room temperature. Slides were again washed with two 5 minute changes of PBS/BSA, and mounted in a solution of PBS/glycerol (1:1). Slides were examined with a U.V. microscope. Percentages of positive and negatively staining cells were calculated.

2.11. HISTOLOGY.

2.11.1. Tissue Preparation.

Lungs from rats treated with wool and grain dusts were removed at 1, 3, 7, and 14 days post exposure and fixed with 10% formol saline at standard pressure, following broncho-alveolar lavage. Tissue was processed and embedded in paraffin wax, and $5\mu\text{m}$ sections cut which were then stained with haematoxylin and eosin. In addition to the earlier time points, lungs were obtained from rats treated with dust for 3 and 6 months and processed in the same manner.

2.12. SCANNING ELECTRON MICROSCOPY.

2.12.1. Preparation of Samples.

BAL cells from dust treated rats were resuspended in F-10 medium (Gibco, Paisley) containing 0.2% BSA to give a concentration of 1×10^6 cells/ml. Thirteen millimeter round coverslips were placed in the bottom of 24-well plates and 1ml of appropriate cell suspension added to each well. Each cell suspension from dusted rats were set up in duplicate and incubated at 37°C for 2 hours. The medium was then removed and each well washed x3 with PBS, after which adherent cells/aggregates were fixed with 2% glutaraldehyde (BDH, Paisley) in PBS for 2 hours.

Coverslips were removed from the wells, and processed for SEM by dehydrating in a graded series of acetone as follows: 10 minutes in each concentration of 10, 20, 50, 70, 90 and 100% with two changes in 90 and three in 100% acetone. Coverslips were critical point dried, mounted on SEM stubs and gold coated. Samples were viewed in a Hitachi scanning electron microscope.

2.13. STATISTICAL ANALYSES.

2.13.1. Cytotoxicity and Detachment assays.

In both assay systems, experiments were carried out three times on separate occasions. All assays were carried out in microtitre plates and had 3 replicate wells for each condition plus controls. Data for all experiments were examined and analyzed using two-way analysis of variance in the Minitab statistical package from which means and standard deviations were obtained. Significant differences between means were tested using student's t-test.

2.13.2. Aggregate formation, TNF production and Lymph node Stimulation.

Experiments were assessed using two or three factor analysis of variance (General Linear Model - Minitab) where experiment was always a factor. This allowed between experiments variation to be taken out and the effect of treatment to be assessed by variance ratio (F) test. Data for repeat experiments were logarithmically transformed, where necessary, to comply with a normal distribution. All analyses of variance were carried out in the normally distributed data. The data presented in the figures is untransformed. When there was a positive

F test, showing a significant global treatment effect, individual means were compared using t tests with a pooled estimate of standard error, as required.

RESULTS.

RESULTS.

3.1. INFLAMMATORY CELL RECRUITMENT AND AGGREGATE FORMATION AFTER ORGANIC DUST INSTILLATION.

3.1.1. BAL Cell Populations.

Scanning electron microscope characterization of the wool dust used in this study showed that in addition to wool fibres, a small amount of plant material was present.

Instillation of 1mg of wool and grain dusts into the rat lung resulted in an initial acute inflammation as assessed by the influx of neutrophils over time points of 1, 3, 7 and 14 days (Figure 1.1). The saline exposed animals showed no neutrophil influx into the BAL at any time point. At day 1, in the wool exposed groups, approximately 70% of the total cells in the BAL were inflammatory neutrophils. The grain-exposed animals produced approximately 30% neutrophils. Three days post exposure, the wool treated animals showed a decrease to approximately 20% neutrophils and grain to about 7%. By 7 days, in both treatments, neutrophil numbers had returned to around control levels. Increased numbers of neutrophils was accompanied by an increase in the total cell number in the dust exposed animals (data not shown).

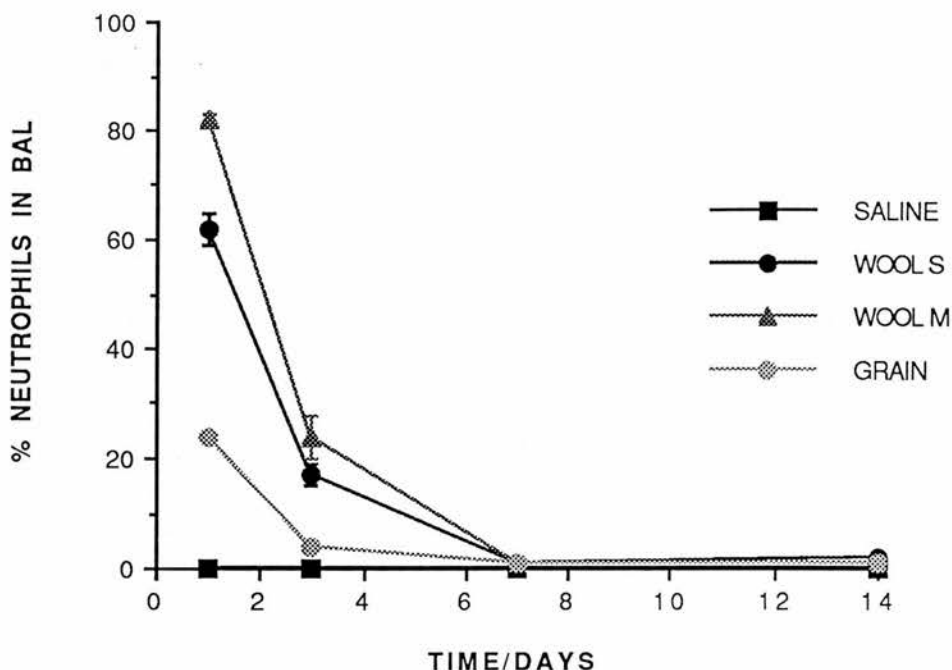


Figure 1.1 The percentage of neutrophils, as a measure of inflammation, in BAL recovered from the lungs of rats instilled with 1mg of dust. Data is the mean and SEM of three experiments.

3.1.2 Aggregates of Cells in BAL.

The BAL of dust-treated rats contained aggregates of mononuclear cells at each time point examined. The aggregates had the light microscope appearance described in materials and methods 2.5.2 and shown in Plate 1. Aggregates were absent from saline treated animals at all time points. At 1 day post exposure, the time point with the greatest number of neutrophils, aggregates of cells also contained neutrophils. The number of aggregates per cytopsin showed a steady increase from day 1, peaking at day 7 and remaining elevated after 14 days (Figure 1.2). The grain-treated animals showed a similar response to that seen in the wool dusted groups, although the magnitude of the response was substantially less. At the 7 day time point, the number of aggregates in the BAL of grain treated rats reached a peak mean of 8 aggregates, which had declined to 4 aggregates per cytopsin at 14 days post exposure.

When aggregates were processed and viewed by scanning electron microscopy they had the appearance shown in Plate 2 Right which contrasts with the lack of aggregates seen in control BAL (Plate 2 Left).

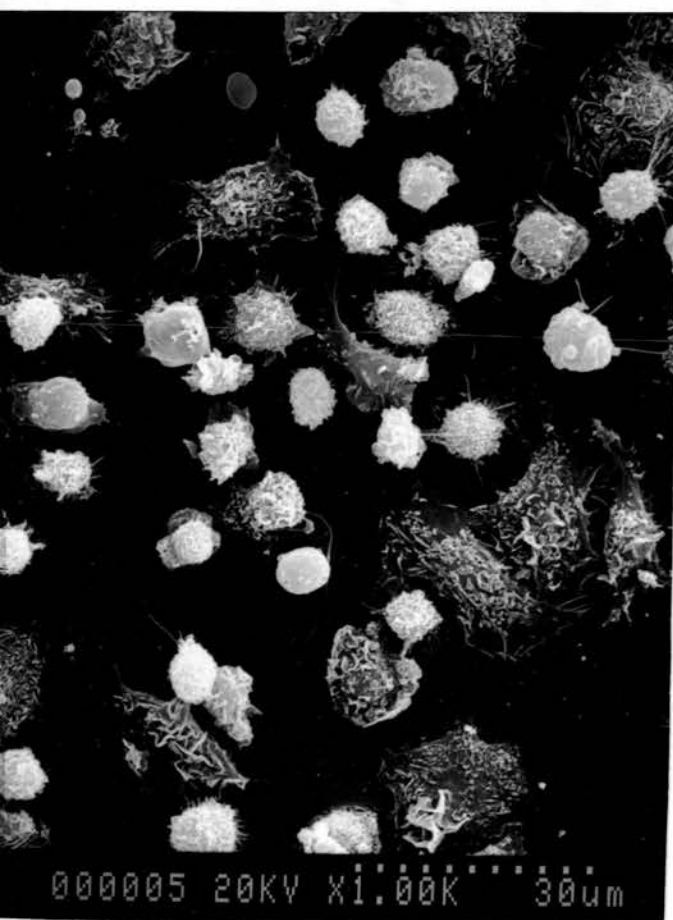


Plate 2 Left.
Control BAL



Plate 3 Right.
An Aggregate.

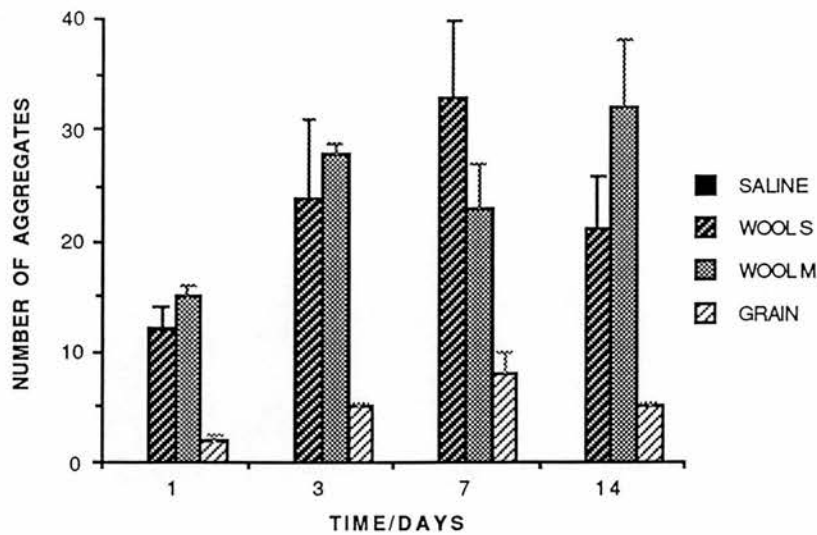


Figure 1.2. The number of aggregates per cytospin from rats treated intratracheally with 1mg wool dust S and M, and grain dust 1, 3, 7 and 14 days post exposure. Results are the mean and SEM from triplicate groups of animals at each time point.

3.1.3 The Effect of Washed Dust and Dust Leachates on Recruitment and Aggregate Formation.

The hypothesis that a soluble product on the wool dust was responsible for the recruitment of inflammatory neutrophils and the formation of aggregates was tested by washing the dust and instilling a) the washed dust, or b) the washings, or leachate, into the rat lungs for a period of 1 day. The results are shown in figure 1.3.

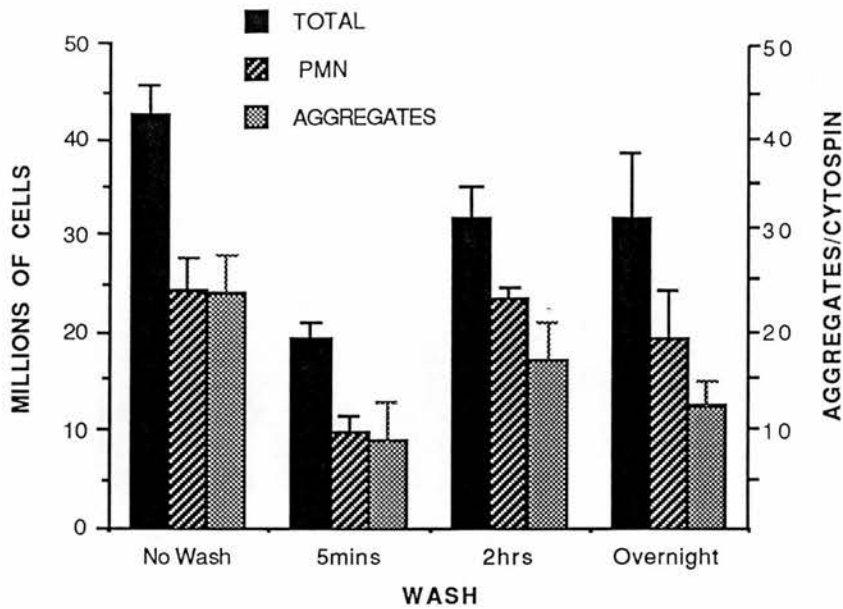


Figure 1.3. The effect of washed wool dust M on the recruitment of inflammatory neutrophils and formation of aggregates after instillation into the lungs for 1 day. Wool dust M was unwashed, or washed for 5 minutes, 2 hours or overnight. One milligram of dust suspended in 0.5ml of PBS was instilled into the lungs. The data is the mean and SEM from three rats per treatment.

The data shows that washing the dust for as little as five minutes was sufficient to reduce the total number of neutrophils and aggregates in the recovered BAL. Washing the dust for 2 hours or overnight slightly reduced the numbers of neutrophils and aggregates, although this was not as efficient as the 5 minute wash.

In a separate experiment, the leachate from dust M was washed for 2 hours or overnight and then instilled into rat lungs for 1 day and the BAL examined.



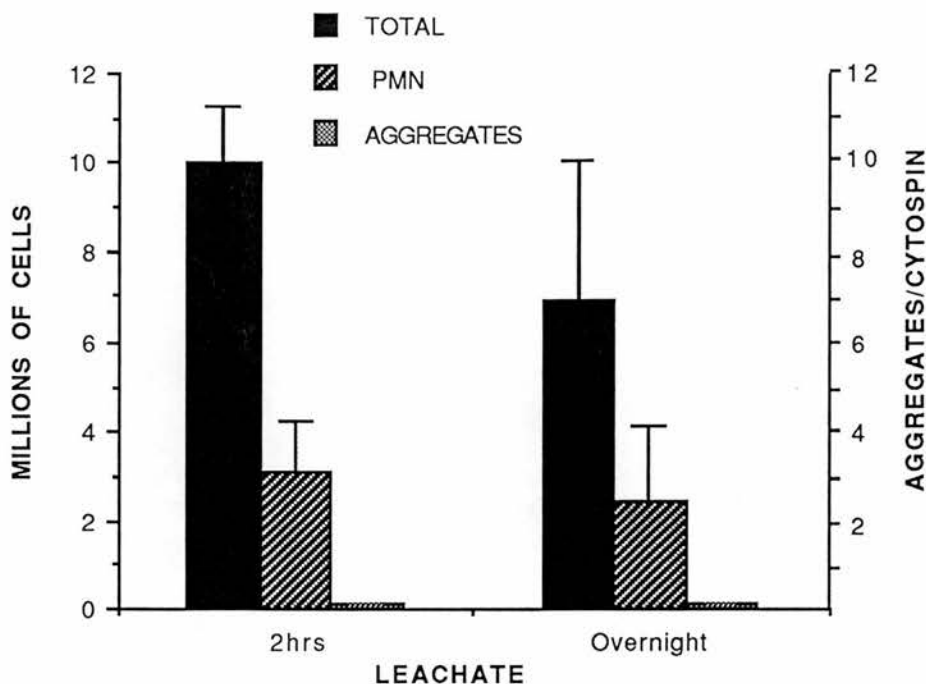


Figure 1.4. The effect of wool dust M leachate from dust washed for 2 hours or overnight on the recruitment of inflammatory cells and formation of aggregates following instillation into the lungs for 1 day. A 0.5ml volume of leachate was instilled, equivalent to the leachates obtained from 1 mg dust.

The data from this experiment shows that leachate from wool washed for 2 hours and overnight (5 minute leachate was lost due to bacterial contamination) was still able to produce neutrophil recruitment into the lungs. Interestingly, virtually no aggregates of cells were produced.

The recruitment of inflammatory neutrophils may be the result of contaminating endotoxin on the wool dust. To test this, we prepared a second leachate from wool dust S and treated half on polymyxin columns to deplete any LPS. The leachate and treated leachate was instilled and BAL carried out 1 day later. The results are shown in figure 1.5.

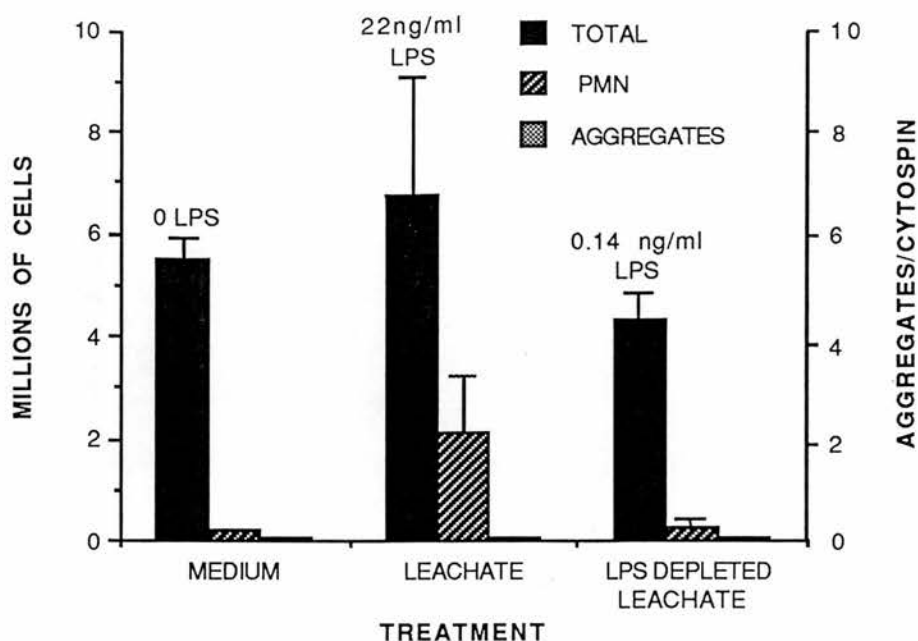


Figure 1.5. The effect of wool dust S leachate and leachate depleted of endotoxin. The graph shows the recruitment of inflammatory neutrophils and formation of aggregates after instillation into the lungs for 1 day. The data is the mean and SEM from three rats per treatment.

The results suggest that a substantial part of the inflammogenicity of wool leachate is due to the presence of bacterial endotoxin. The polymyxin treatment reduced the number of neutrophils to more that half the amount recruited by the whole leachate. There were no aggregates in the whole leachate or depleted leachate.

3.1.4. Effect of Endotoxin *in vivo*.

The lungs of animals which received LPS intratracheally at a concentration of 10µg/ml (5µg per animal) were lavaged 1 day later. There was a substantial inflammatory response, around 75% of the cells in the BAL were neutrophils. As in the dust leachate-treated animals, there were no aggregates present in the LPS-treated animals. There were no aggregates in the saline-treated rats (Figure 1.6).

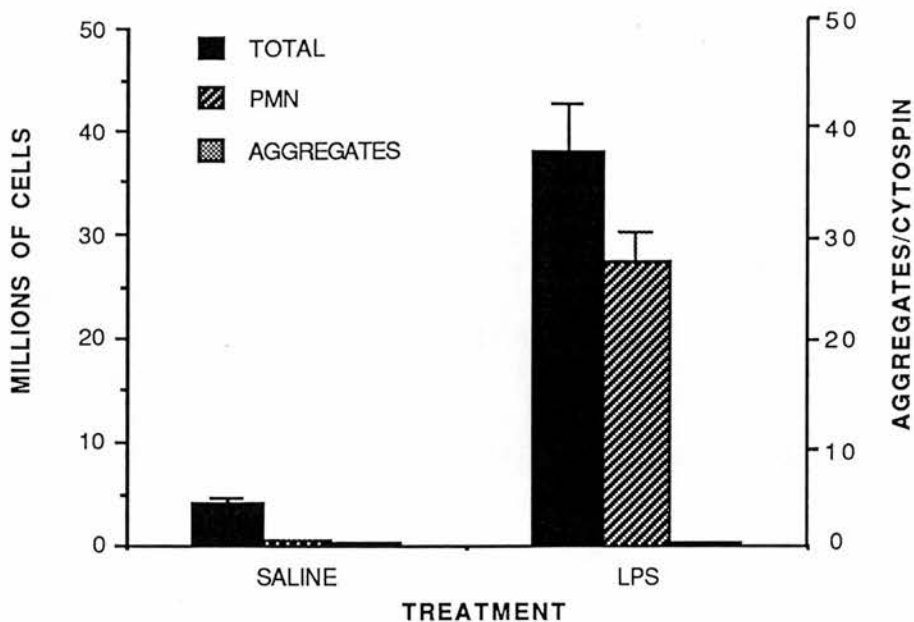


Figure 1.6. The effect of 10µg/ml LPS. The graph shows the recruitment of inflammatory neutrophils and formation of aggregates after instillation into the lungs for 1 day. The data is the mean and SEM from three rats per treatment, and from two experiments.

3.1.5. Characterization of Cells in Aggregates.

Aggregates of cells contained almost exclusively mononuclear cells, except at the 1 day time point, where some of the cells were neutrophils. Characterization of the mononuclear cells was achieved with an immunohistochemical technique using anti-macrophage and anti-Pan T-cell monoclonal antibodies and subsequent staining. Figure 1.7 shows the numbers of macrophages and lymphocytes per aggregate in wool dust-treated animals. The figure shows that each aggregate was made up almost exclusively of macrophages which, like the total number of aggregates per cytospin, reached a peak at the 7 day time point. As in the case of the total number of aggregates per cytospin, at day 14, wool dust S treated animals showed a slight decline from 35 to 25 in the number of macrophages per aggregate. Aggregates from grain treated animals were not characterized.

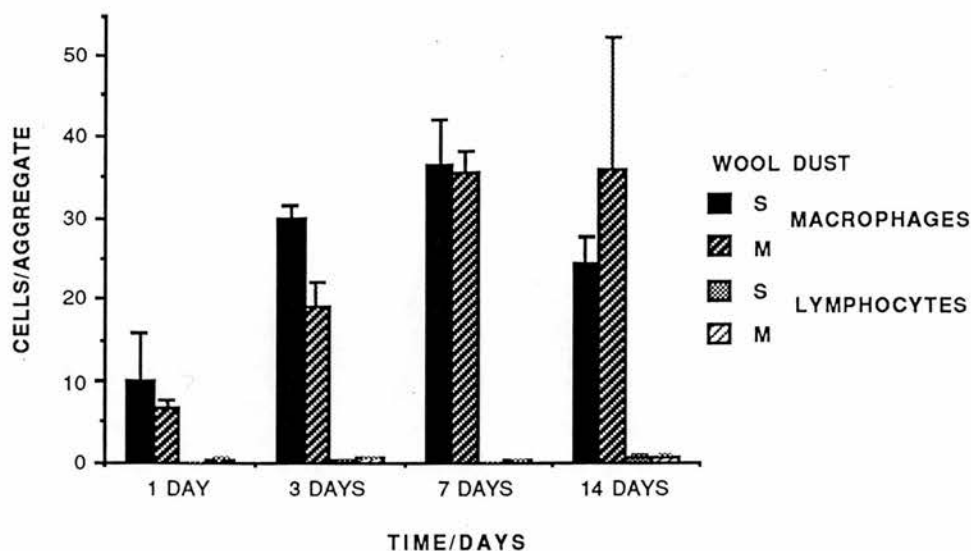


Figure 1.7. The number of macrophages and lymphocytes per aggregate in BAL from rats treated with 1 mg wool dusts S and M. Data is the mean and SEM of ten randomly selected aggregates per cytopspin, in 3 repeated experiments.

3.2. AGGREGATION ASSAY.

3.2.1. Macrophage Aggregation *in vitro*.

To study the aggregation phenomenon observed *in vivo* more fully, an *in vitro* assay was developed where alveolar macrophages from untreated rats were incubated with various treatments. Incubation with medium alone produced some spontaneous aggregation of cells, however, the number of cells comprising each aggregate could be substantially increased by some treatments.

3.2.2. Wool and Grain Dust and Dust Leachate Treatment.

Increases in the number of cells per aggregate were observed after treatment with wool and grain dusts. There was an increase from 18.78 (0.67) cells/aggregate with medium alone, (data is the mean (SEM) of triplicate experiments) to 25.28 (1.03) with wool dust S, and 23.53 (1.25) cells/aggregate with grain dust (Figure 2.1).

The analysis of variance showed a significant effect of treatment ($F=9.52$; $p<0.01$). The individual t-tests showed that all three treatments produced significantly more cells per aggregate than medium alone. Leachates of dust (particle-free extracts) produced no significant change in the number of cells per aggregate compared with medium (Figure 2.1). Medium 13.4 (3.17); Wool S 13.23 (3.34); Wool M 16.4 (6.78); Grain 13.8 (4.41); (Data as mean and SEM). Analysis of variance showed no significant treatment effect ($F=1.34$ $p>0.05$).

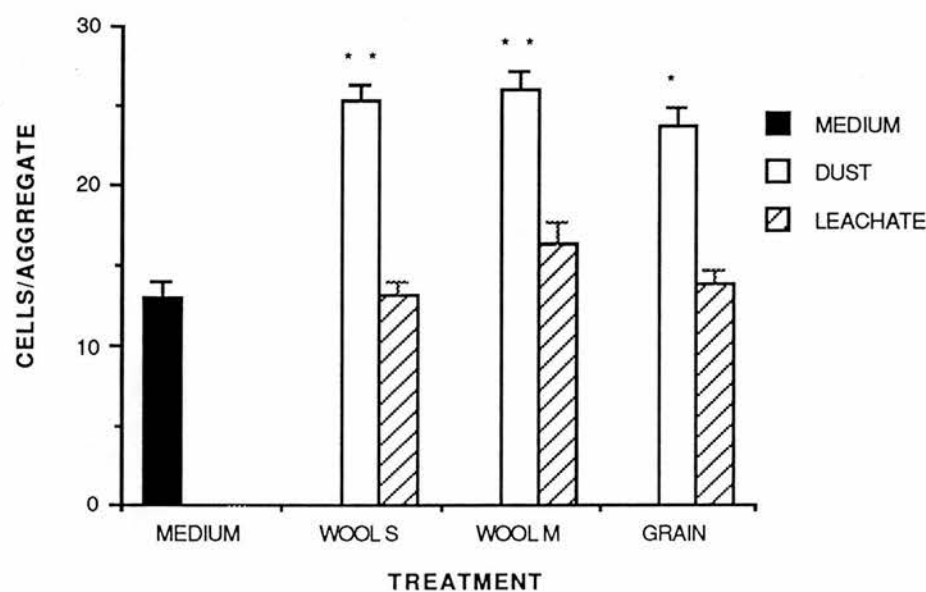


Figure 2.1. The effect of wool and grain dusts and dust leachates on alveolar macrophage aggregation *in vitro*. Significant increases were noted for each dust compared with medium alone. The data is represented as the mean and SEM of the number of cells per aggregate. Ten random fields /treatment were assessed and the experiment was performed on three separate occasions. (**= $p<0.01$; *= $p<0.05$). There was no significant effect of the dust leachates compared with medium.

3.2.3. Macrophage Activating Agents.

Rat alveolar macrophages were treated with endotoxin, PMA, TNF and fibronectin *in vitro*, and aggregation measured (Figure 2.2). In the analysis of variance, a significant treatment effect was observed, ($F=8.88$; $p<0.01$). In individual t-tests, significant ($p<0.001$) differences between medium and endotoxin and PMA were noted. Treatment with TNF produced a smaller but still significant effect ($p<0.05$), whereas fibronectin showed no statistically significant difference.

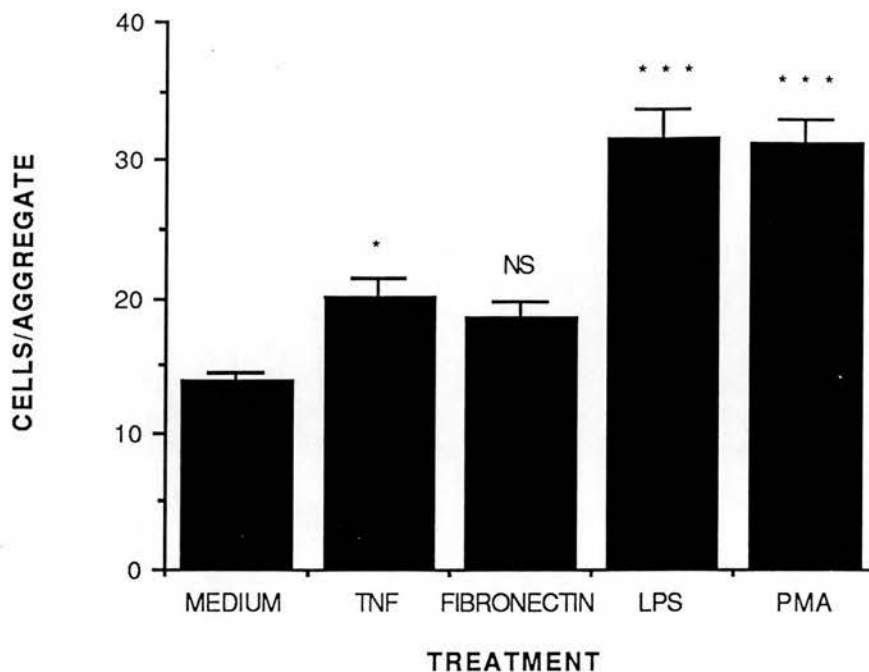


Figure 2.2. In vitro aggregation of rat alveolar macrophages on treatment with TNF, LPS, PMA and fibronectin. The data is the mean and SEM of the number of cells per aggregate. Ten random aggregates per treatment were assessed, and the experiment was repeated on three separate occasions. (*= $p,0.05$; ***= $p<0.001$; NS = not significant, medium control compared with treatment)

3.2.4. Macrophage Supernatants.

Treatment of control alveolar macrophages with macrophage supernatants produced either no change (Wool M, Grain and LPS macrophage supernatants), or slightly decreased the number of cells per aggregate (Wool S). (Table 3).

TABLE 3.

SUPERNATANT	CELLS/AGGREGATE + S.D.	
MEDIUM	14.7	3.06
MACROPHAGES ALONE	18.3	3.27
MACROPHAGES + WOOL S	13.9	3.03
MACROPHAGES + WOOL M	17.6	4.90
MACROPHAGES + GRAIN	18.2	4.52
MACROPHAGES + LPS	18.6	5.40

Table 3. Number of cells per aggregate following treatment of alveolar macrophages with macrophage supernatants *in vitro* (Mean; SD of triplicate wells in one experiment).

3.2.5. Staurosporine Treatment and Macrophage Aggregation.

Activation of macrophages by PMA involves the second messenger Protein Kinase C (PKC). We examined the effect of staurosporine, a PKC inhibitor, on the aggregation of macrophages treated with LPS and PMA (Figure 2.3).

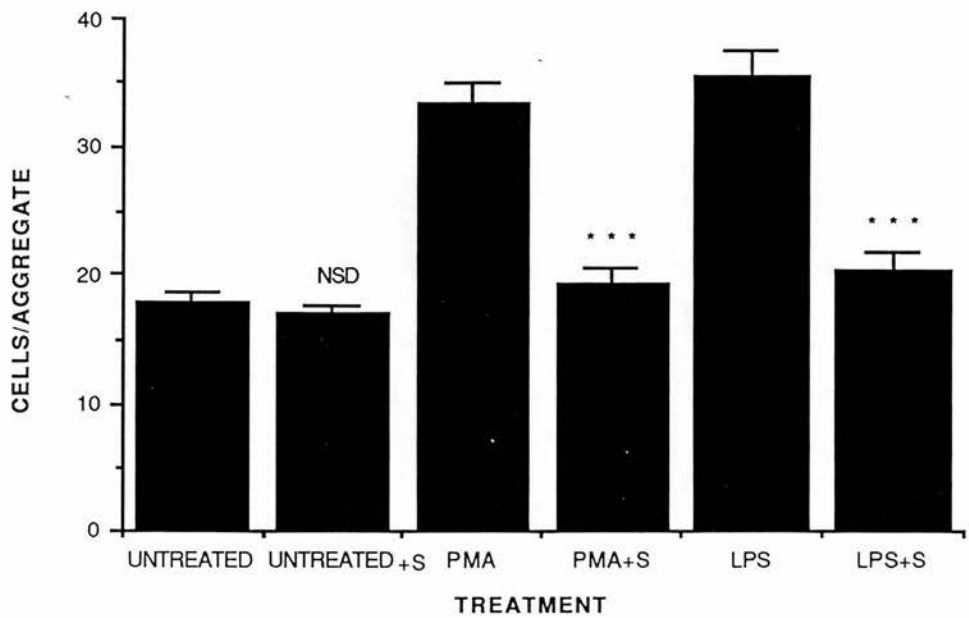


Figure 2.3. The number of macrophages per aggregate in untreated and treated with PMA and LPS with and without the PKC inhibitor staurosporine. The data is the mean and SEM of ten randomly selected aggregates per treatment in three experiments. NSD = No Significant Difference; ***= $p < 0.01$ in comparisons between staurosporine and non-staurosporine groups for each treatment.

Treatment with LPS and PMA produced significantly more cells per aggregate than medium alone ($F=7.12$; $p < 0.05$), and there was also a significant treatment/staurosporine interaction ($F=10.91$; $p < 0.05$). There was no effect of staurosporine on the spontaneous aggregation of macrophages, but cells which had been stimulated with PMA and LPS and treated with staurosporine, were reduced to control values. The activation of PKC by LPS or PMA may result in macrophage stimulation.

In contrast to the effects of staurosporine on PMA and LPS treated macrophages, the inhibitor of PKC produced only a small decrease in organic-dust mediated aggregate formation (Figure 2.4), which was not statistically significant ($F=0.35$; $p > 0.05$). There was no significant interaction between staurosporine and treatment ($F=1.32$; $p > 0.05$).

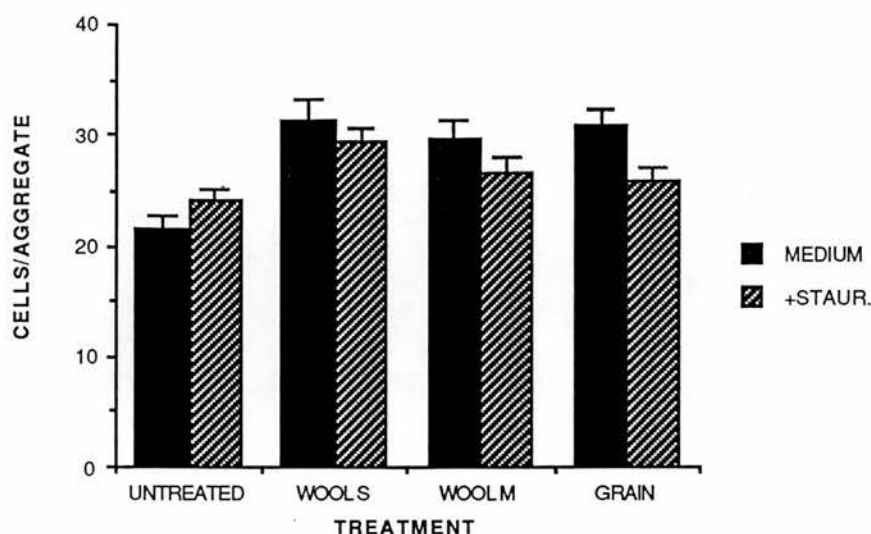


Figure 2.4. The number of macrophages per aggregate in wool and grain treated cells *in vitro* with and without staurosporine. Data is the mean and SEM of ten randomly selected aggregates/treatment, and is the result of three separate experiments.

3.2.6. Role of Divalent Cations.

Removal of calcium and magnesium ions with EDTA resulted in complete abolition of aggregate formation in all treatments and in the medium control. This is consistent with a role for integrin involvement in the aggregation of macrophages.

3.2.7. Effect of Antibodies to LFA-1 and ICAM-1 on Aggregate Formation.

Cells which were treated with LPS along with anti LFA-1 and anti ICAM-1 antibodies; a clear dilution effect was shown (figure 2.5). At a 1:200 dilution of antibody, the number of cells/aggregate began to approach control values. An irrelevant antibody (Pan-T cell, OX 19) had no effect on aggregate formation. By analysis of variance, there was no significant effect of antibody type ($F=2.96$; $p>0.05$), but a significant effect of antibody dilution was demonstrated ($F=35.21$; $p<0.001$).

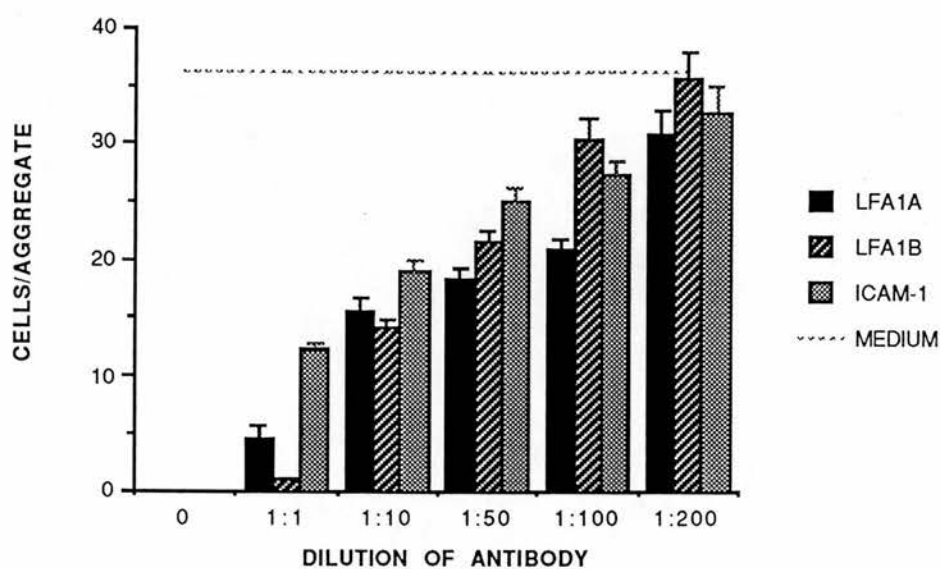


Figure 2.5. Effect of dilution of supernatant on the number of LPS-treated macrophages/aggregate. The data is the mean and SEM of three separate experiments.

Plates 3 and 4 show staining of LFA-1 β adhesion molecules on aggregates of rat alveolar macrophages treated *in vitro* with LPS. The plates show staining of macrophages after initial incubation with mouse anti-rat CD18 antibody and subsequent biotin/peroxidase immunostaining. The illustrations clearly show the presence of CD18 which has a more granular appearance in the higher magnification example.

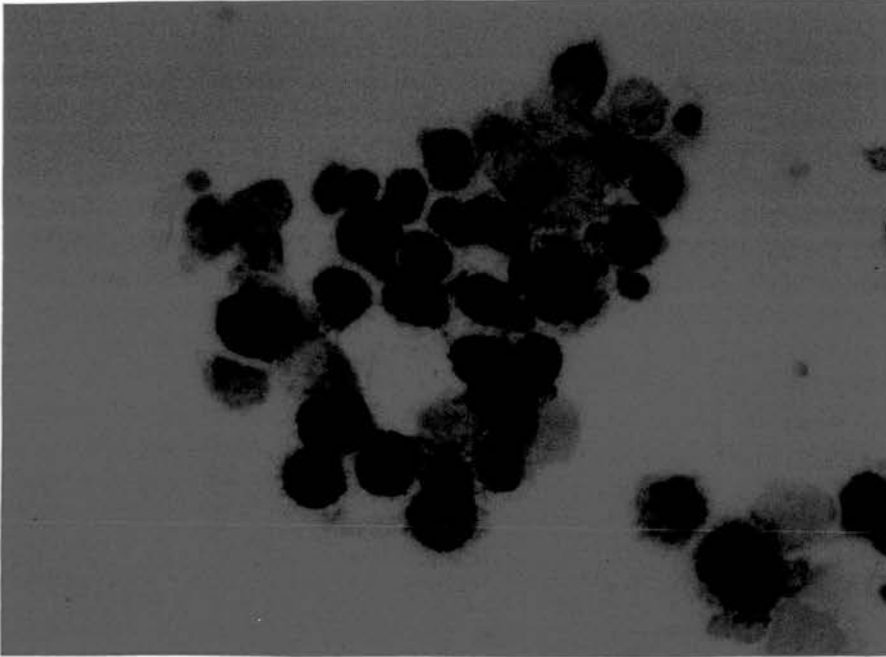


PLATE3. See description above. Original Magnification x200.

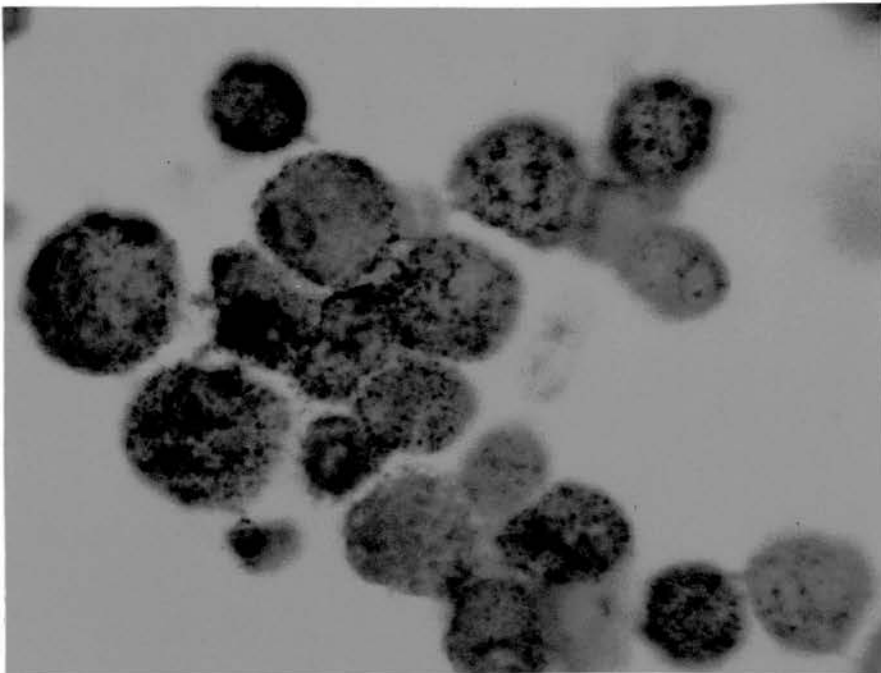


PLATE4. See description above. Original Magnification x400.

3.3. EFFECT OF WOOL AND GRAIN DUSTS ON ALVEOLAR MACROPHAGES.

3.3.1. Wool Dusts S and M.

Treatment of ^{51}Cr -labelled rat alveolar macrophages with wool dust produced no significant cytotoxic effect, measured as release of the radiolabel. The positive control dust DQ12 quartz produced significant lysis of the macrophages (Figure 3.1).

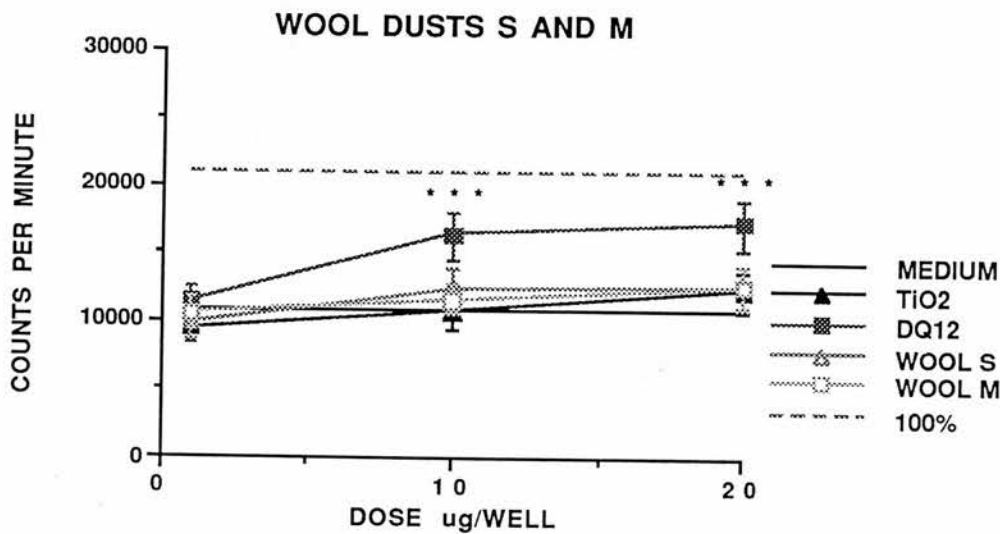


Figure 3.1. The cytotoxic effects of wool dusts S and M on ^{51}Cr labelled rat alveolar macrophages. Injury assessed by monitoring the release of the radiolabel into the culture medium. Data is the mean and SEM of triplicate wells in 3 separate experiments. (***) $p<0.001$ compared with medium alone).

3.3.2. Grain Dust.

As with the wool treatment, treatment of ^{51}Cr labelled rat alveolar macrophages with whole fractions of grain dust produced no significant cytotoxic effect. The positive control dust DQ12 quartz produced significant lysis of the macrophages (Figure 3.2).

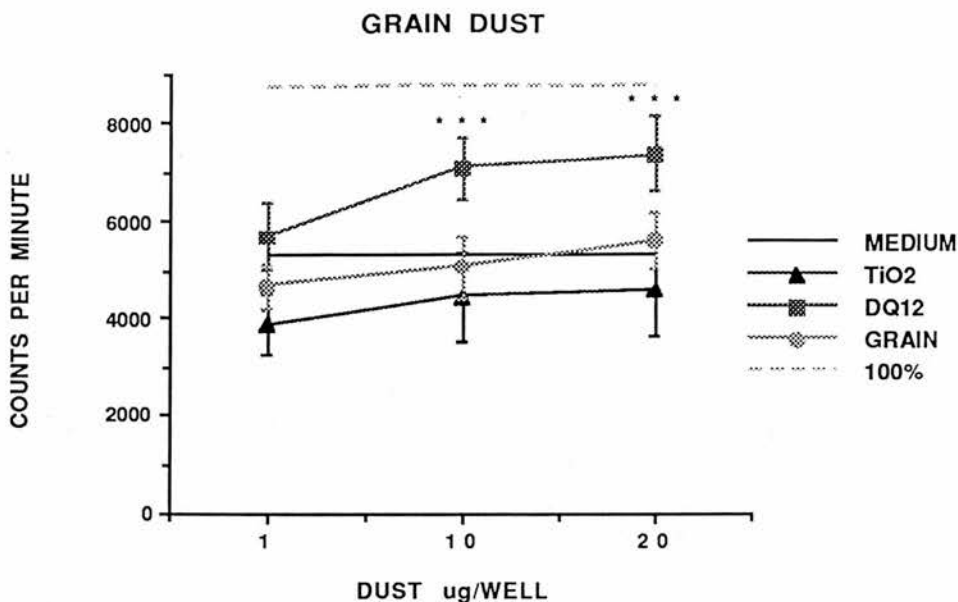


Figure 3.2. The cytotoxic effects of grain dust on ^{51}Cr -labelled rat alveolar macrophages. Injury assessed by monitoring the release of the radiolabel into the culture medium. Data is the mean and SEM of triplicate wells in 3 separate experiments. (** $p < 0.001$ compared with medium alone).

3.4. EFFECT OF WOOL AND GRAIN DUSTS ON A549 EPITHELIAL CELLS.

3.4.1. Whole Dust.

3.4.1A. Lytic Injury.

Inspirable samples of wool dusts S and M, grain dust and the negative control dust TiO_2 produced no significant lytic injury to A549 epithelial cells at any of the doses tested (Figure 4.1A). In contrast, the positive control dust DQ12 quartz and dust collected from surfaces in wool mill S (wool S ledge), produced a significant lytic injury at the higher doses. Fully lysed cells released 11912 cpm of ^{51}Cr in the wool S experiment, and 26893 cpm ^{51}Cr for the wool M experiment.

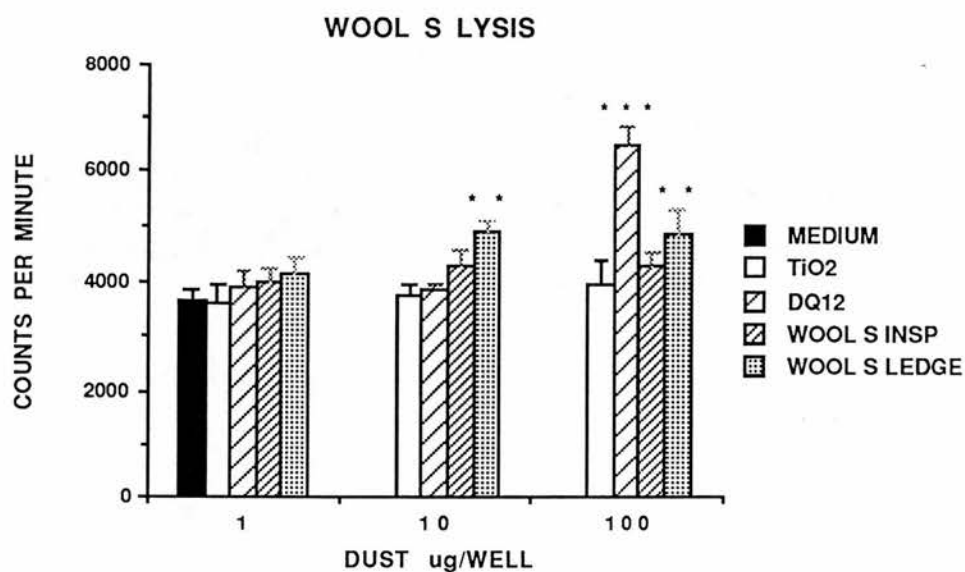


Figure 4.1 A. The lytic effect of wool and grain dusts, and the control mineral dusts DQ12 (quartz), and TiO₂ on A549 epithelial cells. Counts/minute is the release of ⁵¹Cr from labelled cells. Data is represented as the mean and SEM of triplicate experiments. (**p<0.01; ***p<0.001 compared with medium control). Fully lysed cells released 44993 cpm of ⁵¹Cr.

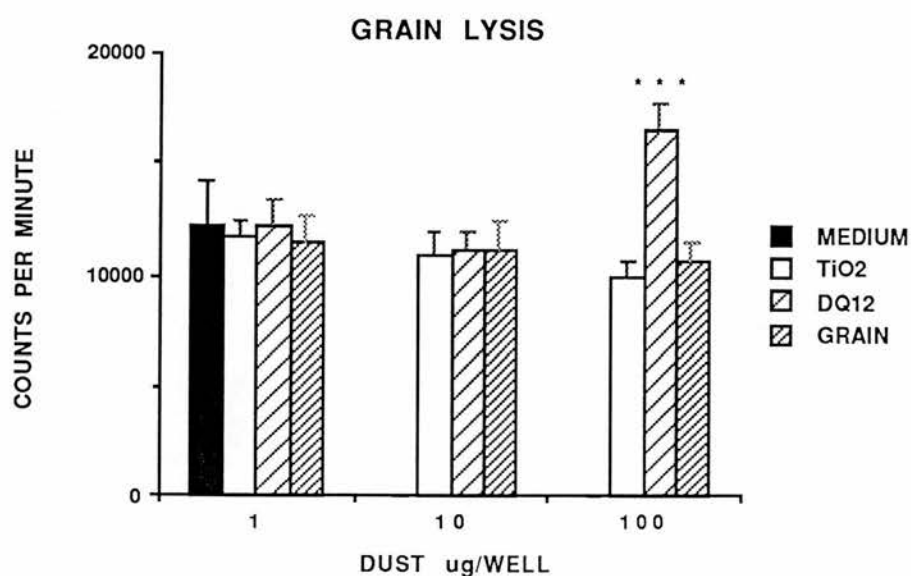
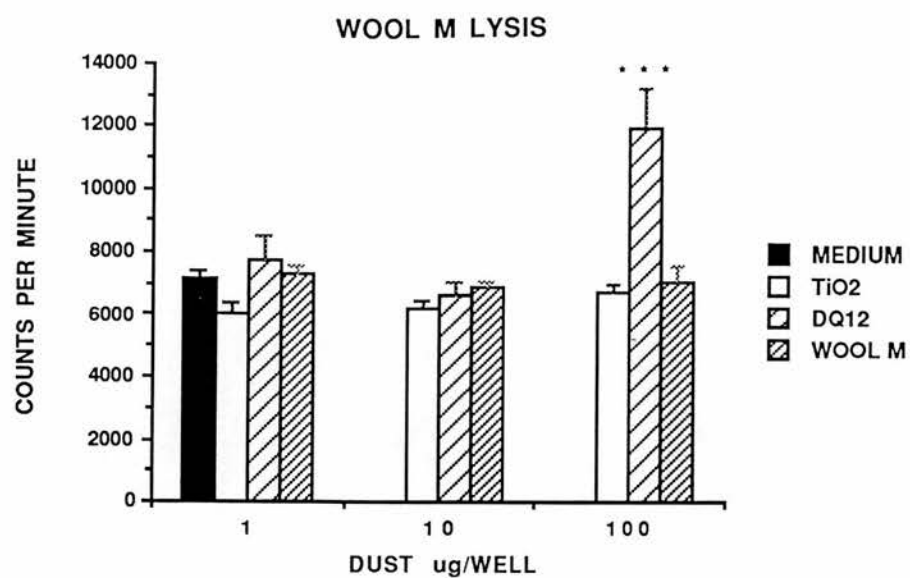


Figure 4.1A continued. See legend on previous page.

3.4.1B. Detachment Injury.

Neither wool dusts, grain dust nor the positive and negative control dusts DQ12 quartz and TiO₂ had no effect on the detachment of A549 cells from the substratum (Figure 4.1B).

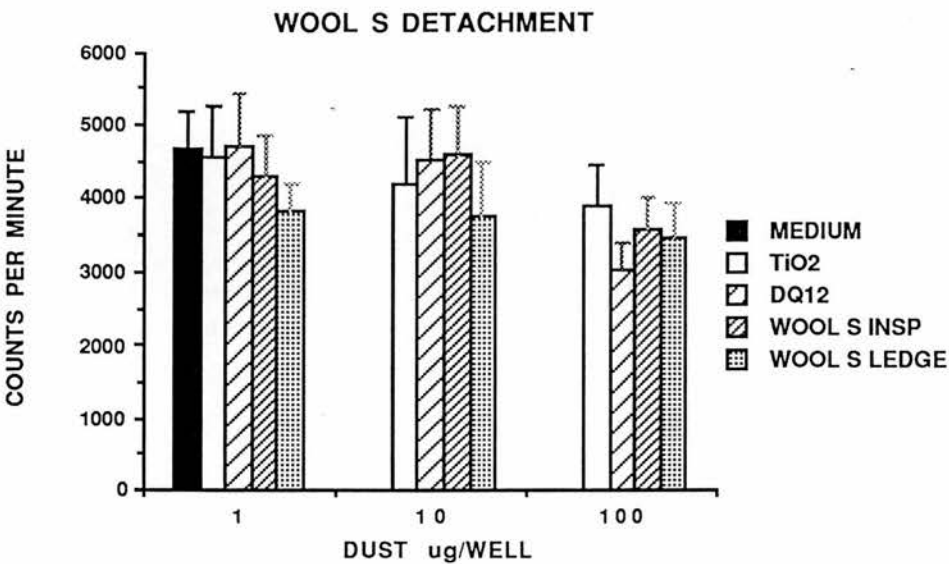


Figure 4.1 B. Detachment of A549 epithelial cells produced by wool and grain dusts, and the control mineral dusts DQ12 quartz, and TiO₂. Counts/minute refers to ⁵¹Cr contained in labelled, detached cells. Data is represented as the mean and SEM of triplicate wells in 3 separate experiments. Detachment of all cells in the wool S group was 8392 cpm ⁵¹Cr, wool M 19744 cpm, and grain 33791 cpm ⁵¹Cr.

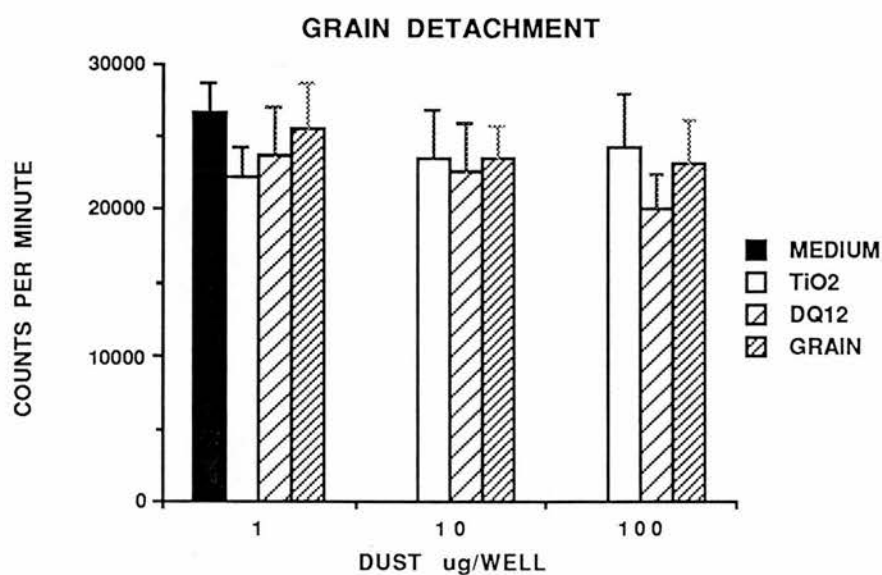
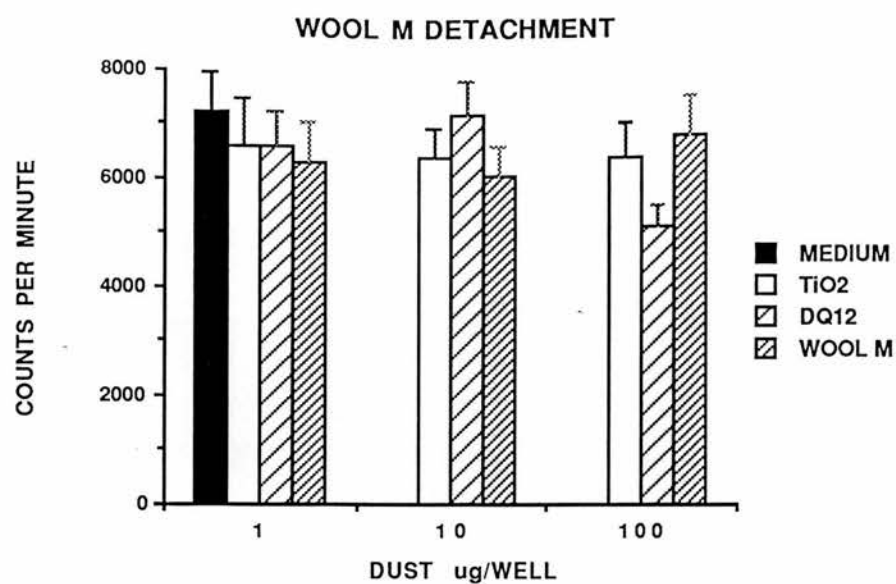


Figure 4.1B continued. See legend on previous page.

3.4.2. Aqueous Extracts of Dust.

3.4.2 A. Lytic Injury.

Extracts of wool dusts S and M and grain dust produced no significant lytic damage to A549 epithelial cells when used undiluted and diluted at various ratios with medium (Figure 4.2 A).

EXTRACTS OF WOOL S AND M - LYSIS EXTRACT OF GRAIN DUST - LYSIS

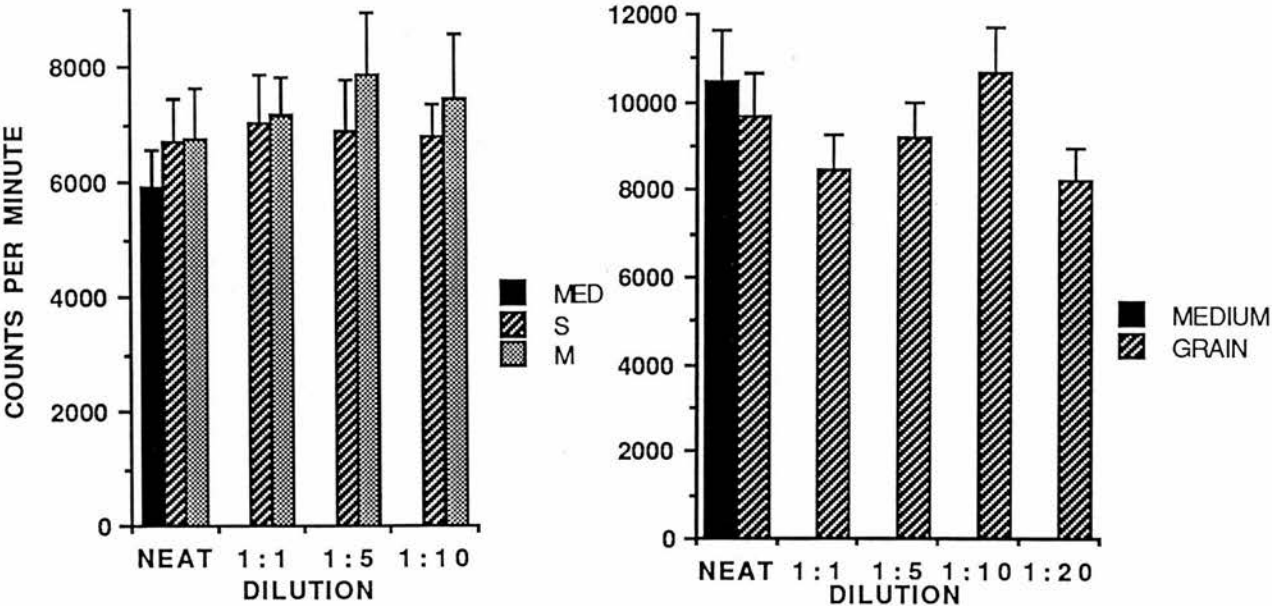


Figure 4.2 A. The lytic effect of wool and grain dust extracts, on A549 epithelial cells. Counts/minute refers to the release of ^{51}Cr from labelled cells. Data is represented as the mean and SEM of triplicate wells in 3 separate experiments. There was no significant difference between the treatments and the medium control. Fully lysed cells released 24505 cpm ^{51}Cr in the wool leachate-treated group, and 42000 cpm ^{51}Cr in the grain extract group.

3.4.2 B. Detachment Injury.

In keeping with the effects produced by whole wool and grain dusts, there was no significant detachment injury produced by aqueous extracts of the dusts (Figure 4.2 B). Detachment produced by grain extract increased with increasing dilution of the extract. Although the effect appeared to be dose dependent, this was not a significant effect.

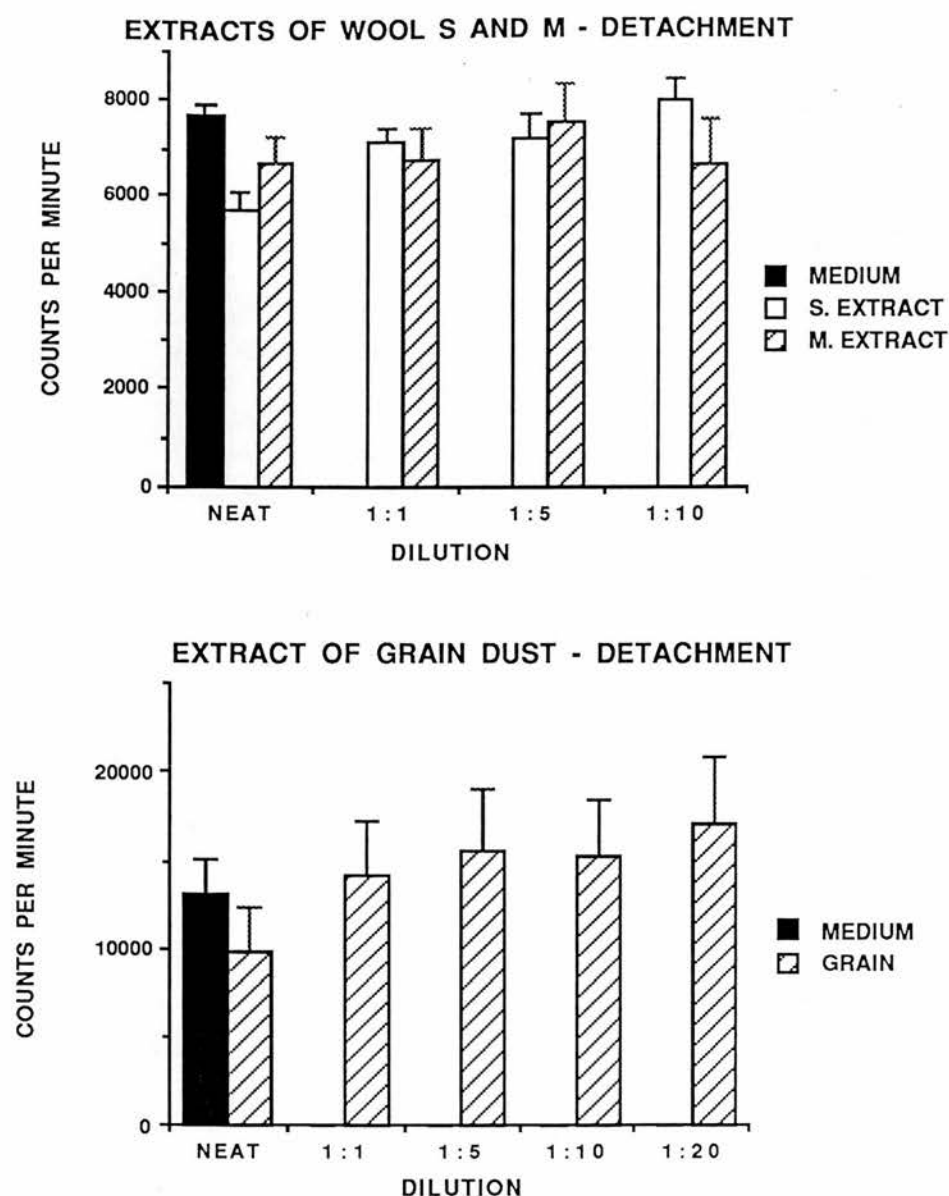


Figure 4.2 B. The detachment of A549 epithelial cells produced by wool and grain dust extracts. Injury was assessed by measuring the Counts/minute of ^{51}Cr in labelled detached cells. Data is represented as the mean and SEM of triplicate wells in 3 separate experiments. There was no significant difference between the treatments and the medium control. Detachment of all cells was 18706 cpm ^{51}Cr in the wool-treated group, and 25200 cpm ^{51}Cr in the grain-treated group.

3.4.3. Endotoxin.

3.4.3 A. Lytic injury.

Endotoxin contamination of organic dusts may be important in some of the deleterious effects in lung function after dust inhalation. Treatment of A549 cells with various concentrations and serotypes of purified endotoxin (Type A 0127:B8; type B 0111:B4; type C 055:B5, Sigma, Poole Dorset) produced no significant lytic injury compared with medium alone, except in the case of endotoxin type C, which showed a significant lytic effect at the highest concentration of 10µg/ml (Figure 4.3 A).

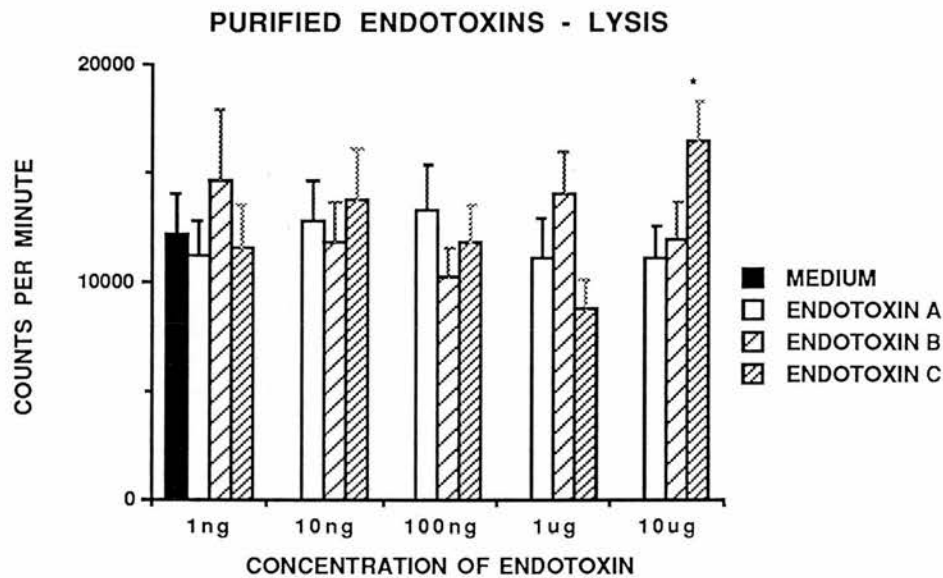


Figure 4.3 A. The lytic effect of three types of endotoxin on A549 epithelial cells. Counts/minute refers to the release of ⁵¹Cr from labelled cells. Data is represented as the mean and SEM of triplicate experiments. There was no significant difference between the treatments and the medium control, except in the case of endotoxin C at the highest concentration of 10µg/ml (*p<0.05). Fully lysed cells released 49526 cpm ⁵¹Cr.

3.4.3 B. Detachment Injury.

There was no significant increase in detachment caused by any of the endotoxins.

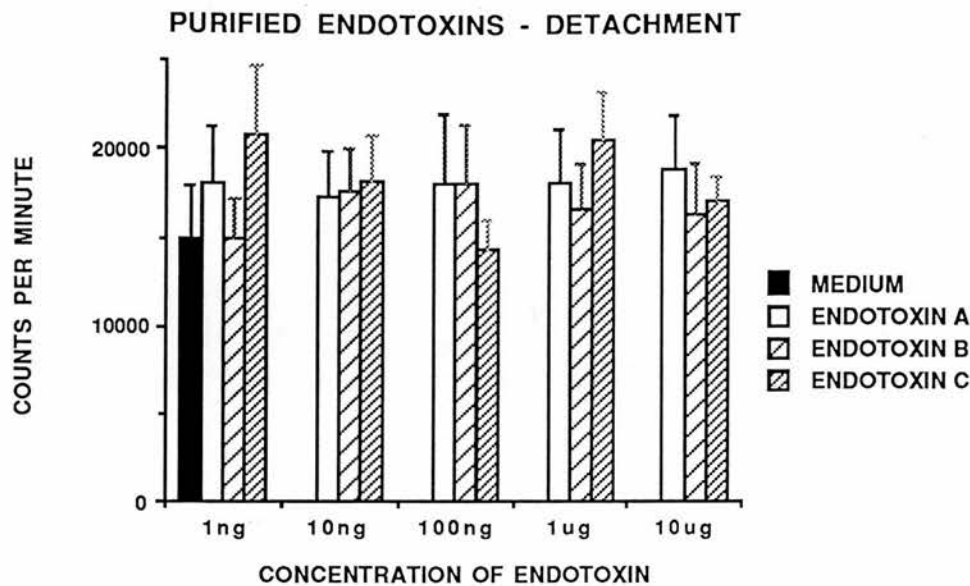


Figure 4.3 B. The detachment injury produced by three types of endotoxin on A549 epithelial cells. Counts/minute refers to ^{51}Cr in detached labelled cells. Data is represented as the mean and SEM of triplicate wells in 3 separate experiments. There was no significant difference between the treatments and the medium control. Detachment of all cells was 37347 cpm ^{51}Cr .

3.5. TNF PRODUCTION.

3.5.1. TNF Production by Rat Alveolar Macrophages Treated with Wool and Grain Dusts *in vitro*.

Alveolar macrophages from untreated rats were obtained by BAL and treated *in vitro* with whole dust, dust leachates, leachates treated with polymyxin, and a standard endotoxin preparation. The supernatants so produced were estimated for TNF content using the standard L929 cell cytotoxicity assay (see methods). The data in figure 5.1 shows TNF production by macrophages treated with whole wool dust samples.

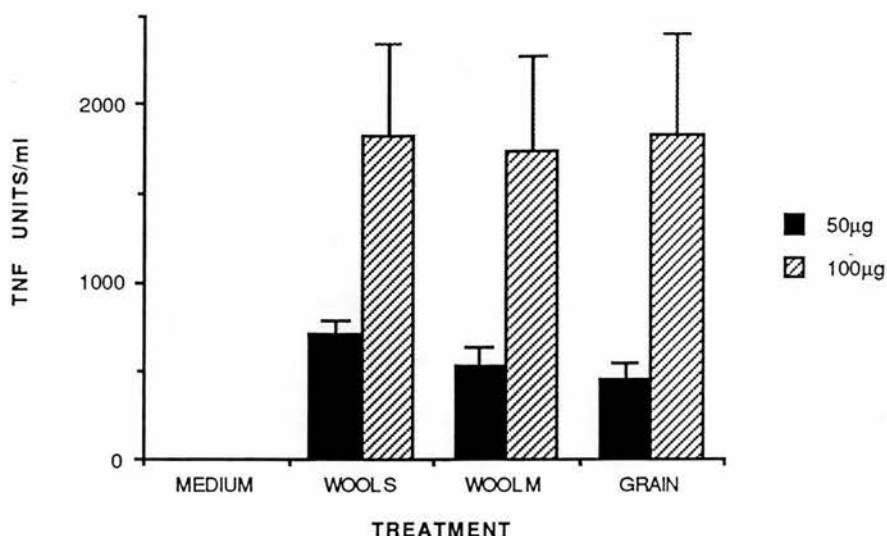


Figure 5.1. TNF production by rat alveolar macrophages untreated or treated *in vitro* with wool dusts S and M, and grain dust. The data is the mean and SEM of the number of units of TNF prepared on 3 different occasions.

The data shows that spontaneous production of TNF in untreated macrophages was virtually nil. In the dust-treated cells, at a concentration of 50µg/ml dust, wool dust S produced approximately 750 Units of TNF/ml, dust M 500 Units TNF/ml, and grain 400 Units TNF/ml. At a concentration of 100 µg/ml the TNF production was approximately 1750 Units TNF/ml in all three cases.

Analysis of variance showed that there was a significant treatment (dust) effect ($F=318.99$; $p<0.001$), a significant dust concentration effect ($F=22.21$; $p<0.01$), and a significant treatment/dose interaction ($F=14.847$; $p<0.001$).

The data shows that wool dust was a potent trigger of TNF production, and that there was a dose dependent effect of dust.

3.5.2. The Effect of Dust Leachates on TNF Production by Rat Alveolar Macrophages.

The possible role of contaminating endotoxin in causing TNF release by alveolar macrophages treated with dust was tested by using dust leachate which had been depleted of endotoxin on polymyxin columns. Figure 5.2 summarizes these results.

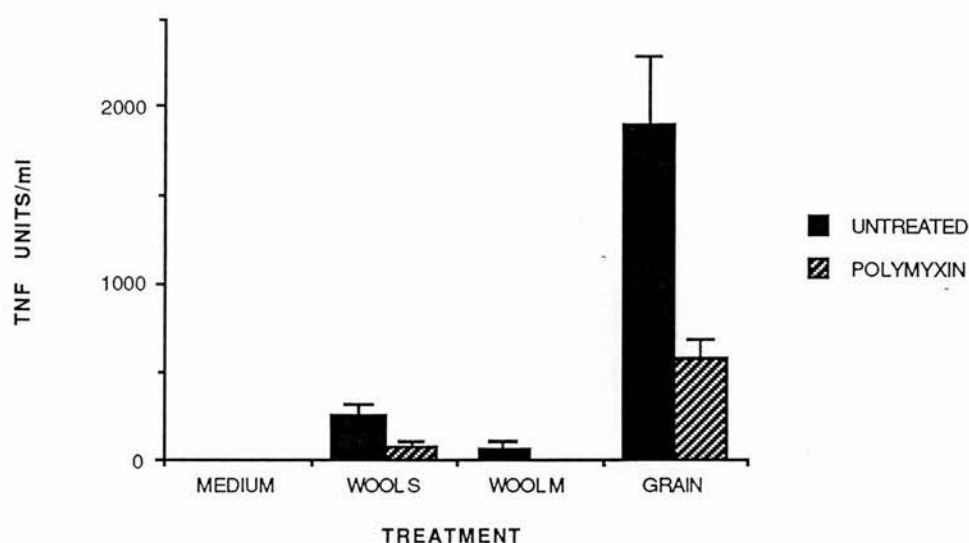


Figure 5.2. TNF production by rat alveolar macrophages treated *in vitro* with wool dust leachate S and M, and grain dust leachate. The graph also shows the effect of endotoxin removal on TNF production. The data is the mean and SEM of the number of units of TNF in supernatant prepared on three different occasions.

The graph shows clearly that untreated macrophages produce no TNF. Wool dust leachates stimulated a modest increase in TNF release, wool S leachate producing about twice as much (approx. 300 Units TNF/ml) as leachate M. The grain leachate, stimulated substantially more TNF secretion than wool leachates, in the range of 2000 Units TNF/ml. Thus, in this assay system, soluble material from grain was more potent than wool in stimulating TNF production by macrophages. Statistically, there was a significant treatment (leachate v medium) effect ($F= 81.65$; $p<0.001$) when leachate was compared with medium alone.

A striking effect was seen in supernatants which had been depleted of endotoxin (see table 4). Leachates treated with polymyxin produced substantially less TNF release by alveolar macrophages than with untreated leachates. The results suggest that the bulk of the TNF production was due to the presence of bacterial endotoxin, there being little or no detectable LPS when leachates post polymyxin were measured by the LAL assay.

TABLE 4.

REDUCTION IN TNF PRODUCTION BY DUST
LEACHATES AFTER POLYMYXIN TREATMENT

LEACHATE	% REDUCTION
WOOL S	66
WOOL M	87
GRAIN	70

Analysis of variance showed in these results that there was a significant effect of polymyxin ($F= 17.08$; $p<0.05$). There was, however, no statistically significant treatment/polymyxin interaction ($F= 0.62$; $p>0.05$).

3.5.3. Effect of Pure Endotoxin on TNF Production by Rat Alveolar Macrophages.

TNF production by macrophages treated *in vitro* with a commercially available endotoxin preparation was assessed to further confirm the role of LPS. Various concentrations of LPS were used, ranging from 0.2 to 25µg/ml to produce supernatants from untreated macrophages; a clear dose-dependent effect was demonstrated (figure 5.3).

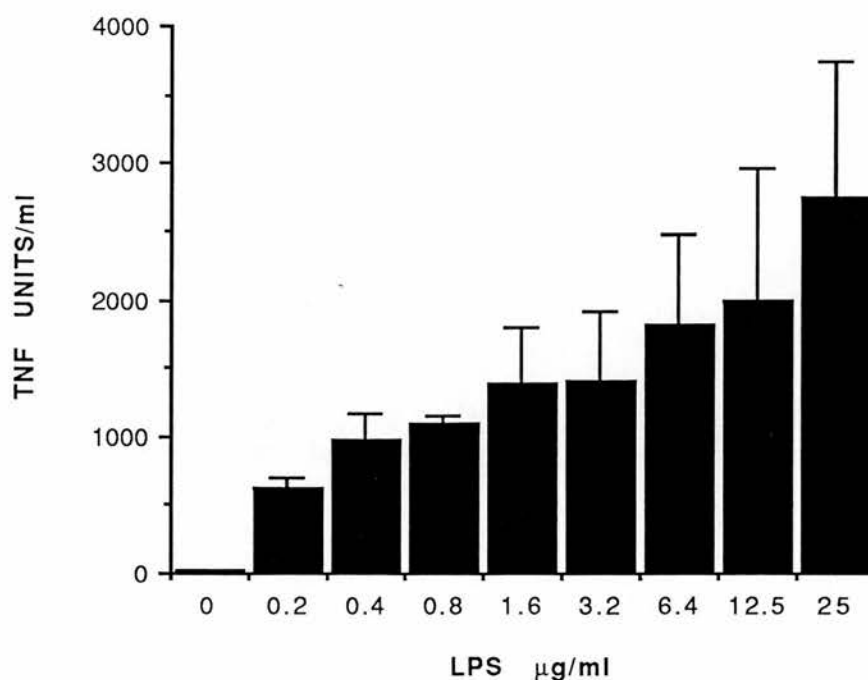


Figure 5.3. TNF production by rat alveolar macrophages treated *in vitro* with various concentrations of endotoxin. The data is the mean and SEM of the number of units of TNF in supernatant measured on three different occasions.

There was virtually no TNF produced in the medium alone condition, ranging to a mean of approximately 2750 Units TNF/ml at the 25 $\mu\text{g/ml}$ LPS concentration. Statistically, there was a significant effect of LPS dose ($F= 12.20$; $p<0.001$).

3.5.4. Effect of Dust Leachates and Depleted Leachates on TNF Production by Macrophages from C3H/HEJ. Mice.

Mice of the strain C3H/HEJ are non-responders to endotoxin due to a genetic defect; C3H mice respond normally to endotoxin. Peritoneal macrophages from both these strains were treated with dust leachates and endotoxin-depleted leachates to compare the role of endotoxin in TNF secretion. These effects are summarized in figure 5.4.

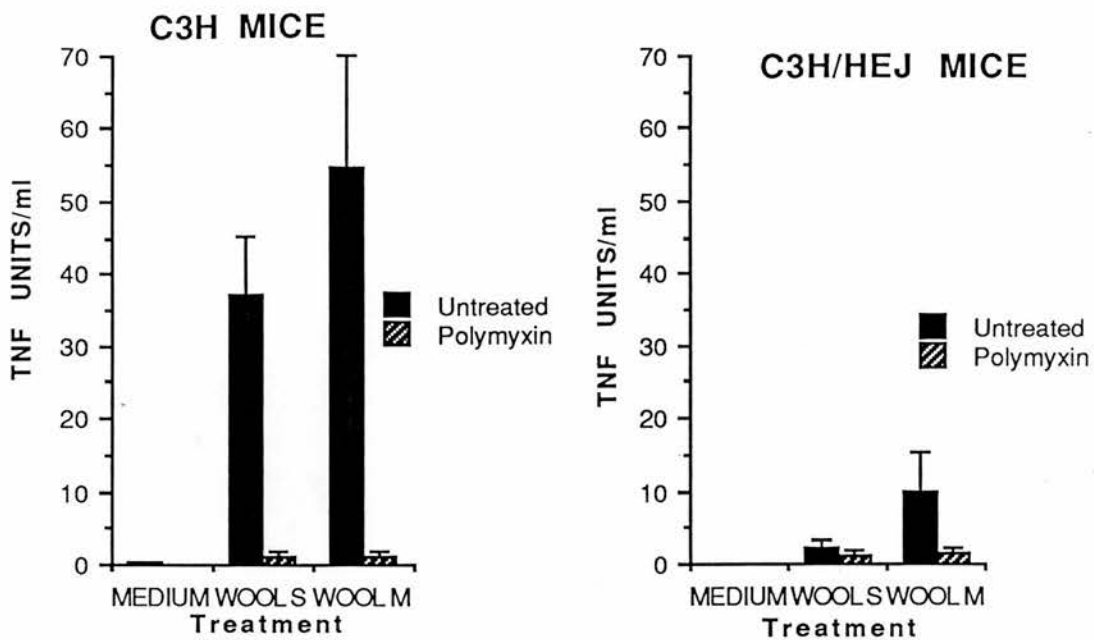


Figure 5.4. TNF production by C3H (normal endotoxin responders) and C3H/HEJ (endotoxin non-responders) peritoneal macrophages treated with dust leachates and leachates depleted of endotoxin. The data is the mean and SEM of triplicate measurements in three separate supernatants.

Figure 5.4 shows that dust leachates can trigger the release of TNF from normal responder C3H mice, similarly to that seen with rat alveolar macrophages. Removal of endotoxin caused a dramatic reduction in the TNF produced (35 Units TNF reduced to 3 Units TNF for leachate S, and 55 Units TNF reduced to 4 Units TNF for leachate M). Grain leachate was not assessed due to the difficulty in preparing a bacteria-free supernatant.

In contrast, in the non-responder C3H/HEJ mouse peritoneal macrophages, very small amounts of TNF were produced, the maximum was 10 Units TNF in untreated leachate M, and approximately 3 Units TNF in untreated leachate S. Removal of endotoxin reduced these responses still further, to 2 Units TNF for both leachates.

In analysis of variance, there was a significant treatment effect (medium v leachate) ($F=15.89$; $p<0.05$), a significant effect of polymyxin ($F=57.70$; $p<0.05$), a significant difference between the two strains ($F=80.43$; $p<0.05$), a significant treatment/strain interaction ($F=73.71$; $p<0.001$), and a significant treatment/polymyxin/strain interaction ($F=31.36$; $p<0.001$).

Collectively, the secretion of TNF by wool and grain dusts and their leachates is due mainly to the presence of contaminating bacterial endotoxin, although additional unknown factors are also present which can stimulate secretion.

3.6. LYMPH NODE CELL RESPONSES.

3.6.1. Characterization of Lymph Node Cells.

Immunostaining of lymph node cells from saline and wool dust-treated rats showed the profile summarized in table 5. In both treated groups at both time points of 1 and 7 days post-exposure, there were between 60% and 70% T-lymphocytes; the anti-macrophage staining showed that approximately 10%-20% of the cells were positive. When the lymphocyte subsets were stained (CD4 and CD8), approximately 40%-50% were positive for the CD4 marker in the saline-treated rats at both 1 and 7 days post-exposure. The dust-treated rats, however, gave only 19% positively staining cells at 1 day, and 51% positive cells at 7 days. Staining for the CD8 marker gave broadly similar results, with 29% and 18% positively staining cells in the saline group at 1 and 7 days respectively. The dusted animals gave 16%-18% positively staining cells at both 1 and 7 days post-exposure.

TABLE 5.

	1 DAY		7 DAYS	
	SALINE	WOOL	SALINE	WOOL
PAN-T	67%	69%	74%	62%
ANTI-MAC	12%	11%	18%	19%
CD4	49%	19%	46%	51%
CD8	29%	16%	18%	18%

Table 5. The number of T-lymphocytes and their sub-sets and macrophages in lymph nodes from rats treated with saline or 1mg of wool dust 1 and 7 days post-instillation.

3.6.2. Effect of Organic Dust on Lymph Node Lymphocyte Responses.

One milligram of wool dusts S and M, and grain dust were instilled into rat lungs. After 7 days, the mitogen-enhanced proliferation of lymphocytes from the draining

lymph nodes was measured. Figures 6.1 - 6.3 shows the effect of the dusts compared with a saline control. The 7 day time point was chosen because it was this time point which produced maximum numbers of aggregates in the BAL, and was a time point which suggested some immune system involvement (see section 1).

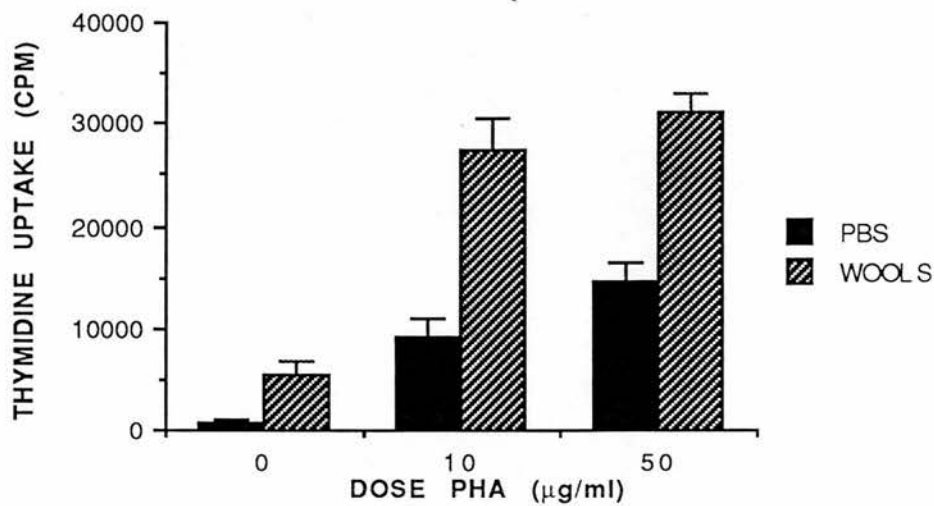


Figure 6.1. The effect of intra-tracheal instillation of 1mg of wool dust S, 7 days previously, on the thymidine uptake of mitogen stimulated lymph node cells compared with a saline control. The data is the mean and SEM of triplicate wells from three rats per dose of PHA performed on three separate occasions.

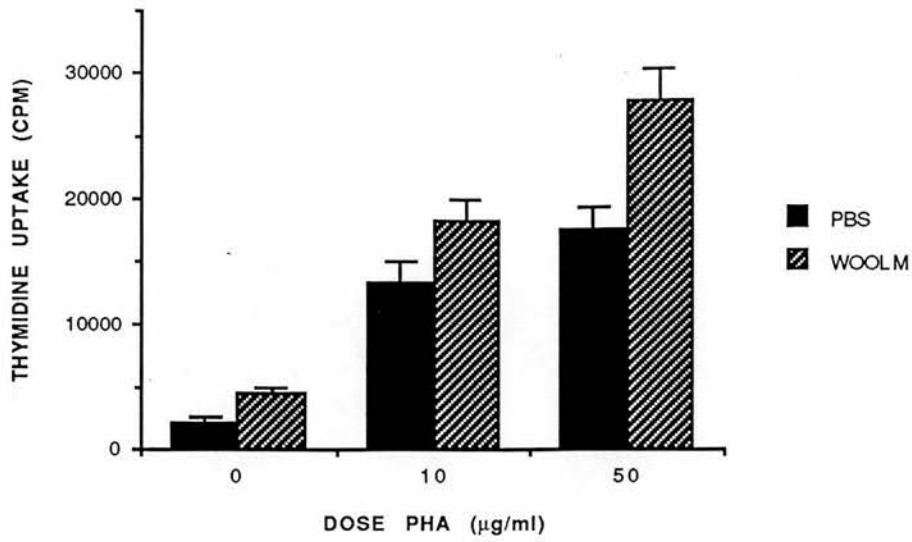


Figure 6.2. The effect of instillation of 1mg of wool dust M, 7 days previously, on the thymidine uptake of mitogen stimulated lymph node cells compared with a saline-instilled control. The data is the mean and SEM of triplicate wells from three rats per dose of PHA performed on three separate occasions.

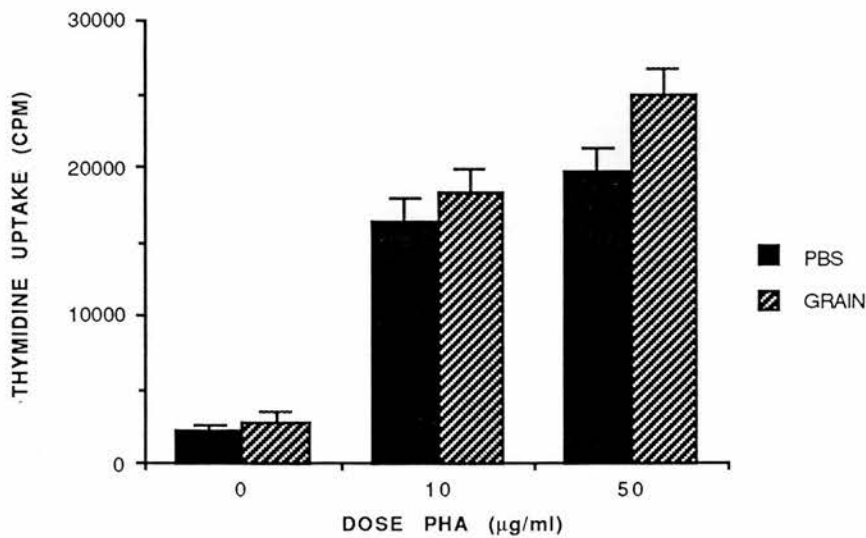


Figure 6.3. The effect of instillation of 1mg of grain dust, 7 days previously, on the thymidine uptake of mitogen-stimulated lymph node cells compared with a saline-instilled control. The data is the mean and SEM of triplicate wells from three rats per dose of PHA performed on three separate occasions.

In all three dust treatments, there was a significant increase compared with the saline control, in the spontaneous proliferation (no PHA) and in the mitogen-enhanced which also showed a dose response.

The results from the analysis of variance table showed that there was a significant effect of dust treatment ($F= 8.94$; $p<0.05$), although no difference between the dusts themselves. There was also a significant effect of dose of mitogen ($F= 76.25$; $p<0.001$).

3.6.3. Time Course of the Effect of Wool Dust on Lymph Node Lymphocyte responses.

Wool dust S was used to investigate the effect of time on thymidine uptake by lymph node lymphocytes. Wool dust S (1mg) was instilled and lymph node cells obtained 1, 3, 7 and 14 days later. Figure 6.4 shows the response to PHA of lymph node lymphocytes from rats instilled with saline or 1mg wool dust.

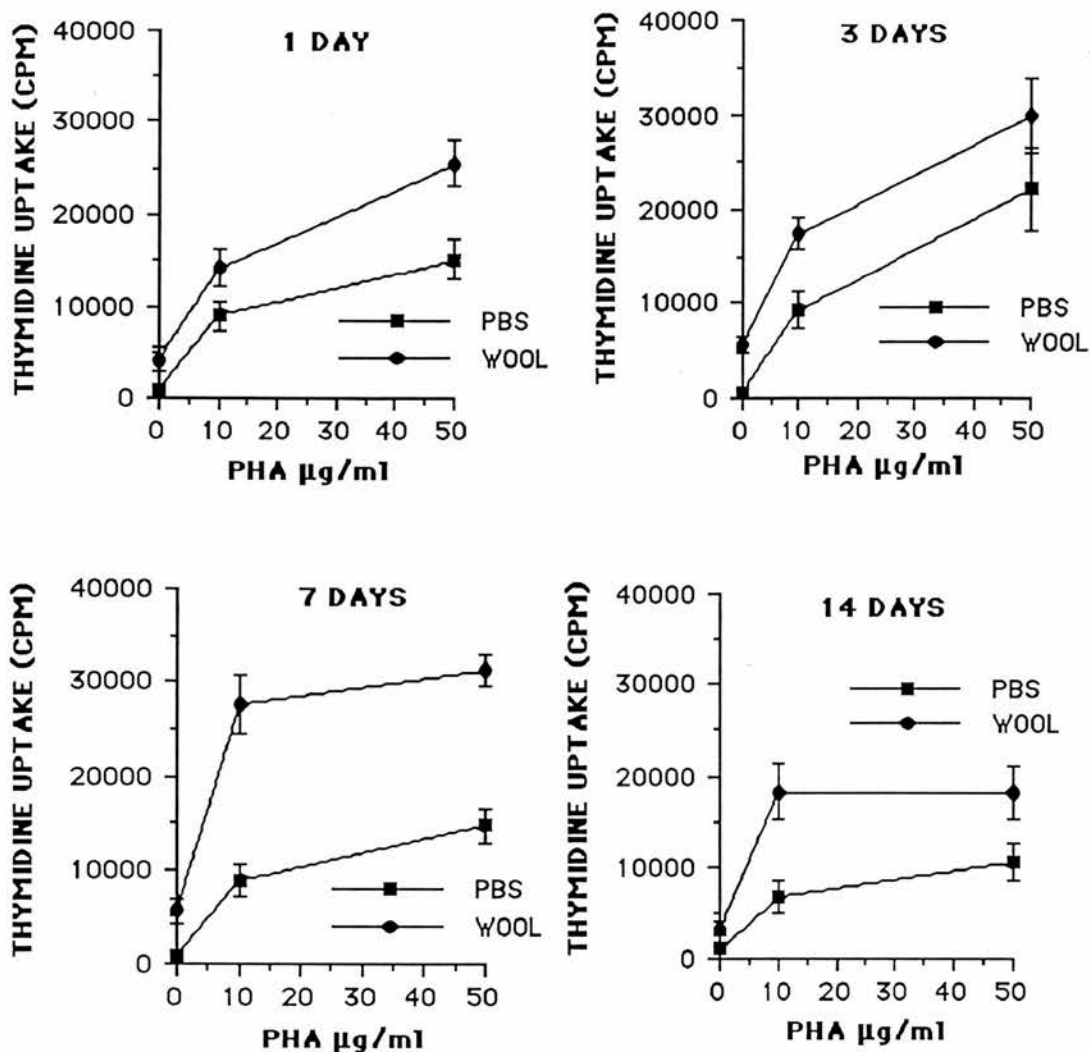


Figure 6.4. Lymph node lymphocyte thymidine uptake after instillation of 0.5ml PBS or 1mg wool S dust into rat lungs. The time course of the response. The data is the mean and SEM of triplicate wells from three rats per dose of PHA performed on three separate occasions.

These experiments show that in keeping with the previous experiment, there was an increase in lymph node cell proliferation in the dust treated animals when compared with the PBS control in the spontaneous proliferation (no PHA) and in the mitogen treatment, which again demonstrated a dose response. This effect was clearly shown at each time point.

The analysis of variance table showed a significant treatment effect between PBS and wool dust S ($F = 126.91$; $p < 0.01$). There was a significant dose effect of mitogen ($F = 118.87$; $p < 0.001$). There was, however, no significant effect of time ($F = 0.80$; $p > 0.05$).

3.6.4. The Effect of an Inert Particle on Lymph Node Lymphocyte Responses.

As an additional control for the effect of inert particles on the thymidine uptake of lymph node lymphocytes, titanium dioxide was instilled into rat lungs; 7 days post exposure the lymph nodes were examined for thymidine uptake in the presence and absence of PHA (Figure 6.5).

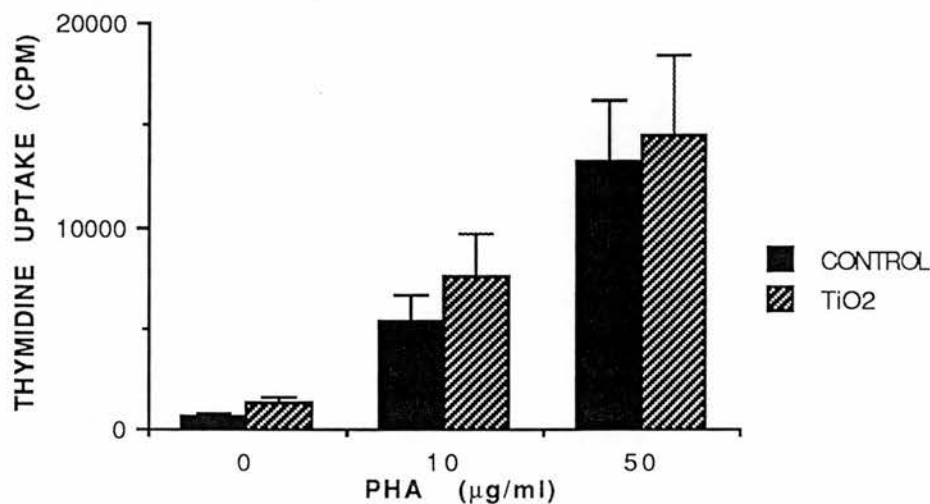


Figure 6.5. The effect of instillation of 1mg of TiO₂ 7 days previously on the thymidine uptake of mitogen-stimulated lymph node cells compared with a saline-instilled control. The data is the mean and SEM of triplicate wells from three rats per dose of PHA performed on three separate occasions.

Analysis of variance showed no significant effect of the different instillation treatments ($F= 0.04$; $p>0.05$). There was, however, a significant effect of dose of mitogen ($F= 16.21$; $p<0.05$).

3.6.5. The Effect of Dust Leachate on Lymph Node Lymphocyte Responses.

An aqueous extract of wool dust S was used to investigate the possible effect of a soluble product present in or on the wool dust surface could have on lymph node lymphocyte thymidine uptake. An aqueous extract was prepared (see methods) instilled into rat lungs, and the lymph node responses were examined 7 days post exposure for comparison with the data shown above. The results are shown in figure 6.6.

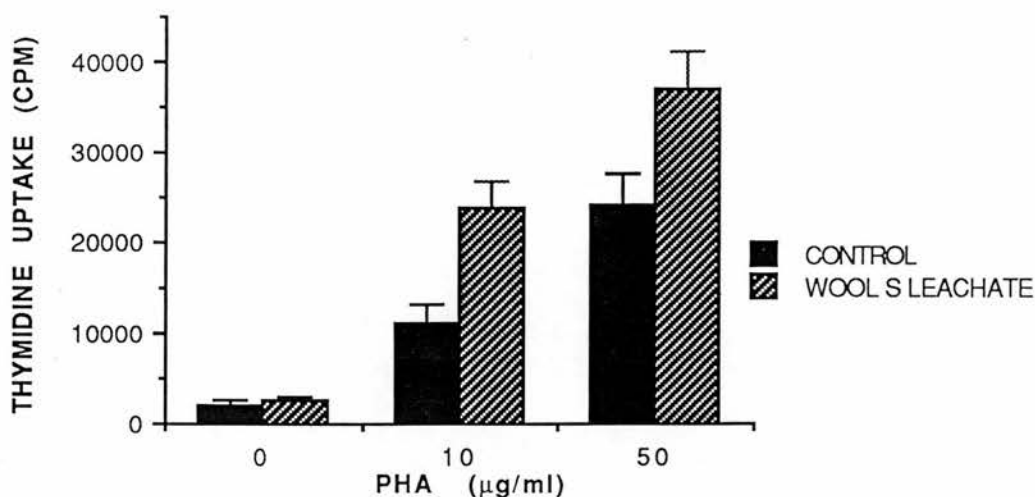


Figure 6.6. The effect of instillation of wool dust S leachate 7 days previously on the thymidine uptake of mitogen-stimulated lymph node cells compared with a saline-instilled control. The data is the mean and SEM of triplicate wells from three rats per dose of PHA performed on three separate occasions.

The analysis of variance showed no significant effect of treatment (control v leachate) ($F = 0.87$; $p > 0.05$). There was a significant effect of dose of mitogen ($F = 28.32$; $p < 0.01$).

3.6.6. The Effect of Washed and Unwashed Wool Dust on Lymph Node Lymphocyte Responses.

An aliquot of wool dust M was washed for 5 minutes or 2 hours in PBS. The leachate from each of these treatments was removed. The washed dust and leachate from each time point, and an unwashed sample was instilled into rats for a period of one day, 1mg dust per rat. Figure 6.7 shows the effect of the dusts on lymph node cell thymidine uptake.

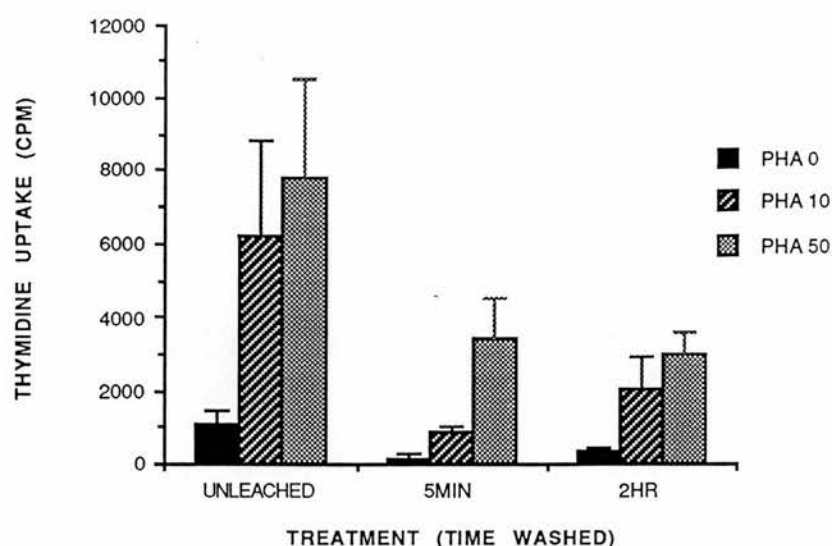


Figure 6.7. The effect of washed wool dust M on lymph node cell thymidine uptake 1 day post instillation. The data is the mean and SEM of triplicate wells, three rats per treatment.

The data presented in figure 6.7 show that washing the wool dust for periods as short as 5 minutes and 2 hours substantially reduced the thymidine uptake by lymph node cells both in the unstimulated and stimulated with PHA.

The analysis of variance for the experiments showed that there was no significant difference between the unwashed and washed dusts ($F=1.298$; $p>0.05$).

3.6.7. The Effect of Endotoxin on Lymph Node Lymphocyte Responses.

The acute inflammatory effect of endotoxin on recruitment of inflammatory cells has been described (section 1). This effect was examined in the lymph nodes of rats instilled with $10\mu\text{g}$ of LPS for 1 day, and the data is shown in figure 6.8.

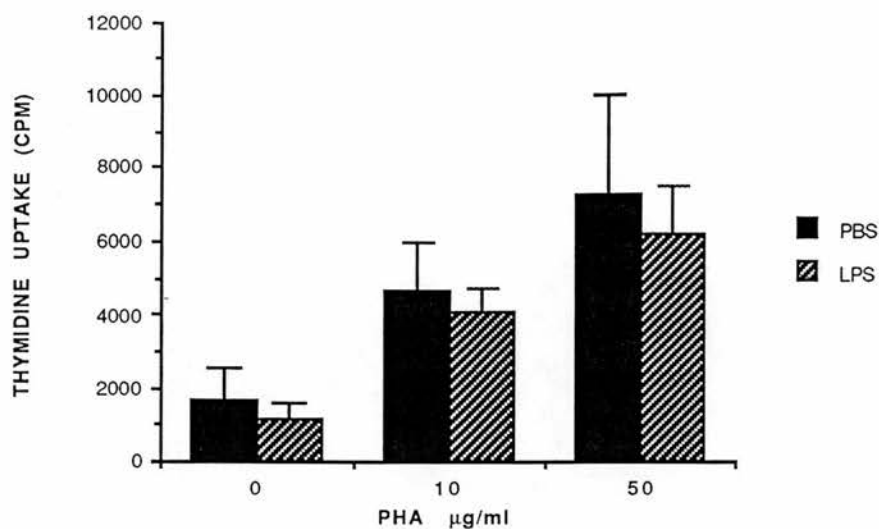


Figure 6.8. Thymidine uptake of lymph node cells from rats treated by instillation of 10 µg LPS or PBS 1 day previously. The data is the mean and SEM of triplicate wells from three rats per treatment.

The data in figure 3.6.8 shows that there was no significant effect of a relatively large dose of LPS on thymidine uptake by lymph node cells 1 day post instillation ($F=0.007$; $p>0.05$). There was a significant effect of dose of mitogen ($F=45.02$; $p<0.0005$) as demonstrated in previous experiments.

An amount of endotoxin equivalent to the amount present in 1mg of wool dust was instilled into rat lungs and the lymph node responses ascertained 7 days later (figure 6.9).

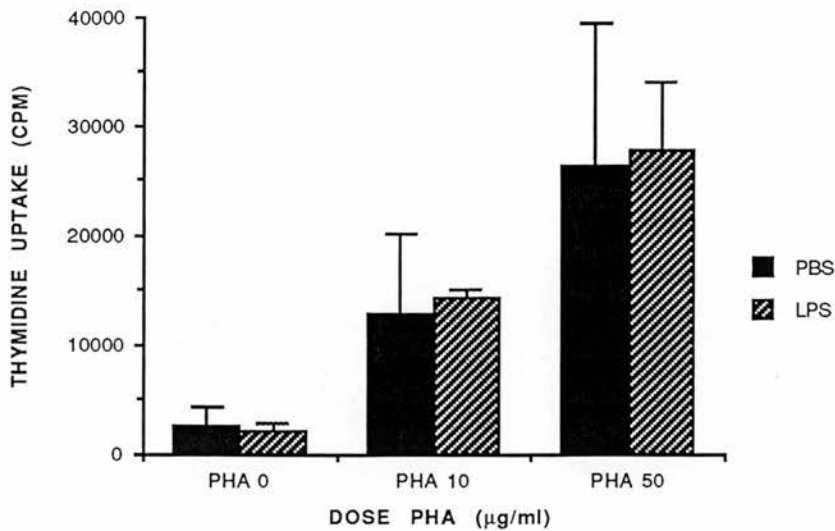


Figure 6.9. Thymidine uptake of lymph node cells from rats treated by instillation of LPS or PBS 7 days previously. The data is the mean and SEM of triplicate wells from three rats per treatment carried out on two separate occasions.

The analysis of variance showed that there was no effect of treatment (LPS v PBS) ($F=0.007$; $p>0.05$). As in the previous proliferation experiments, there was a significant effect of PHA ($F= 222.84$; $p<0.01$).

3.7. HISTOLOGY.

Plates 5-8 show the effect of a single instillation of 1mg of wool dust S into rat lung 3 months post-exposure.

Plate 5 shows a typical granuloma comprising a central area where giant cells and epithelioid cells are clearly visible. The peripheral area of the lesion is thickened and, in the airspaces outwith the lesion, mononuclear cells and aggregates of cells are present.

Plate 6 shows granuloma formation with similar architecture to the lesion represented in plate 5. This illustration contains evidence of epithelial injury (top of photograph) where an entire length of the epithelium has become detached from the substratum.

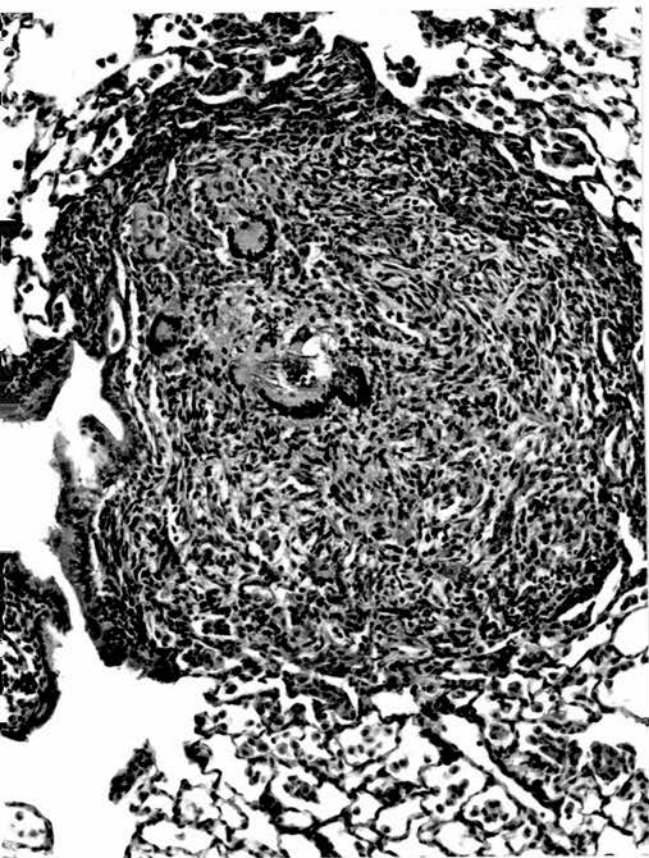


Plate 5. Lungs of rats treated
with 1mg wool dust
3 months post-instillation.
Original Magnification x200.

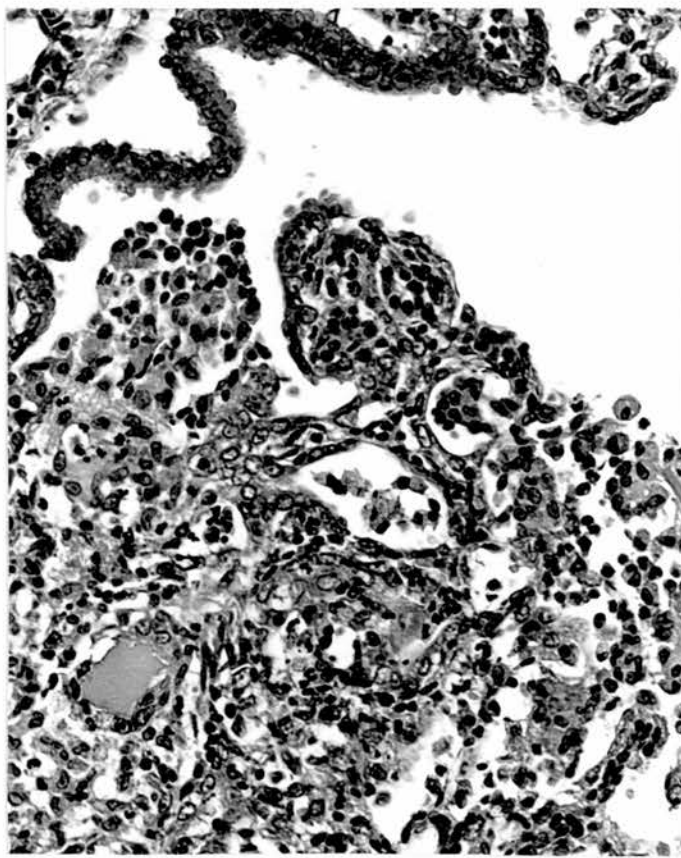


Plate 6. Lungs of rats treated
with 1mg wool dust
3 months post-instillation.
Original Magnification x400.

Plate 7 clearly shows giant cells and aggregates of mononuclear cells in the formation of a granulomatous lesion. The illustration demonstrates the destruction of normal lung tissue.

Plate 8 shows airway wall thickening and evidence of mononuclear cell infiltration. Areas of the airway epithelium has become detached from the substratum. The surrounding lung tissue shows derangement of normal lung tissue.

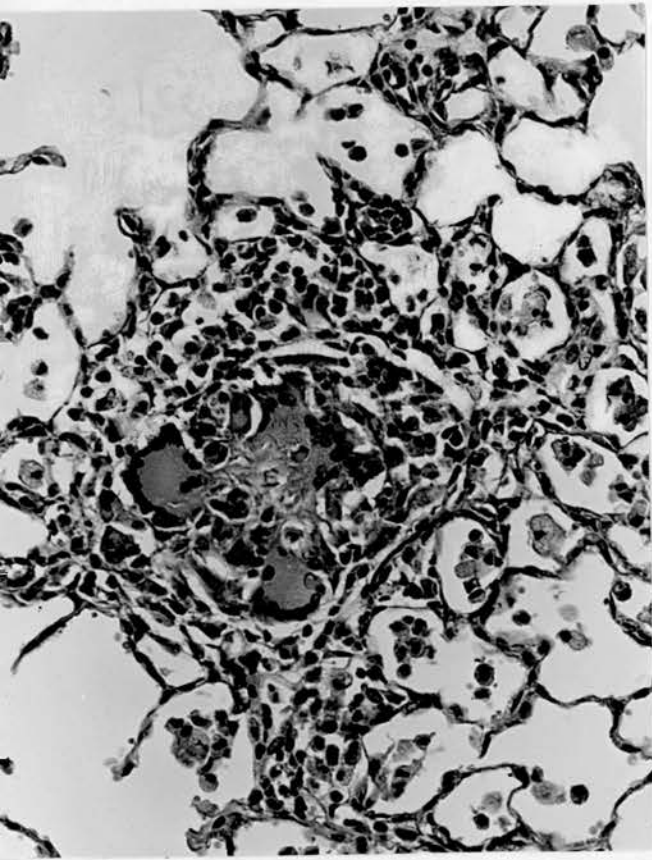


Plate 7. Lungs of rats treated with 1 mg wool dust 3 months post-instillation.

Original Magnification x400.

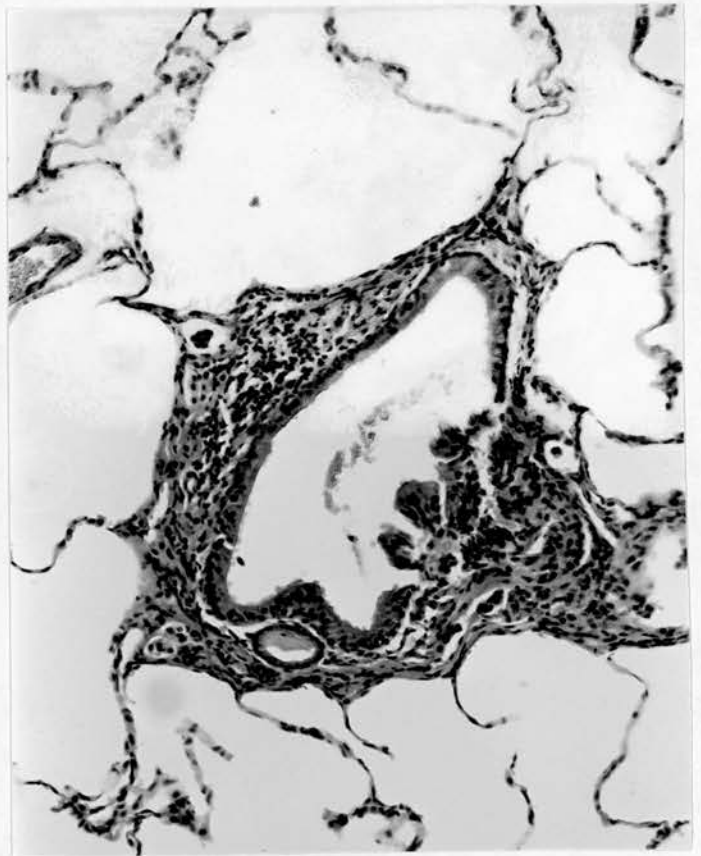


Plate 8. Lungs of rats treated with 1 mg wool dust 3 months post-instillation.

Original Magnification x200.

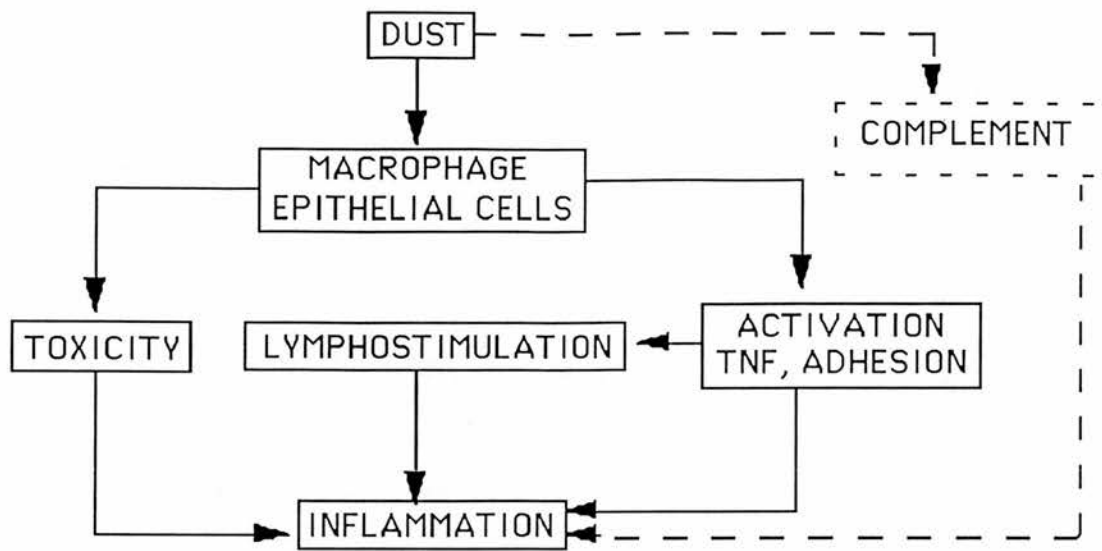
DISCUSSION.

DISCUSSION.

4.1. General.

The purpose of this study was to investigate the mechanism whereby wool and grain dusts could cause lung disease in workers exposed to these dusts. Employees in wool textile mills in the north of England have in a previous study reported symptoms of chronic bronchitis, breathlessness and wheeze (Love et al, 1988). The frequency of these respiratory symptoms were shown to be related to the airborne mass concentration of dust to which the workers were exposed (Love et al, 1988). A similar situation is seen in grain handlers where prolonged exposure to grain dust is associated with chronic bronchitis with or without airways obstruction (Becklake et al, 1980). Dust was collected from the air of wool mills and grain dust was sifted to attain a fine fraction and these were used in a rat model to investigate the likely origin of the inflammation.

A hypothesis was formulated that these symptoms resulted from one or several consequences of deposited organic dust. Bronchitis is inflammation of the airways, which could result from direct injury to epithelial cells and alveolar macrophages. The inflammation produced may be enhanced by the release of cytokines such as tumor necrosis factor from activated macrophages and perpetuated by the activation of cell adhesion molecules. The immune system could also be involved since these are potentially antigenic dusts. These events are summarized schematically in diagram 1. The dust also has the potential to activate complement and has been described previously for wool mill dust but this was not further addressed in the present thesis.



— — — — Not in the scope of this thesis.

Diagram 1.

4.2. Inflammatory Effects of Organic Dust in the Lung.

The normal BAL population of the rats used in these studies was 100% alveolar macrophages. Neutrophils are absent from the BAL of control rats. Whole wool and grain dusts caused inflammation when instilled into rat lungs (Donaldson et al, 1990). We have shown here that soluble extracts of wool and grain dust were active in causing neutrophil recruitment when instilled into the lungs. Much of this activity was attributable to bacterial endotoxin which was present on the dusts and in the dust leachates. Thus, leachates from wool and grain dusts which had been depleted of endotoxin by treatment with polymyxin, produced substantially less recruitment of inflammatory neutrophils. In addition to inflammation whole dust caused aggregates of broncho-alveolar cells to be formed (Donaldson et al, 1990) and these were again evident in this study; leachates alone never produced aggregates when instilled.

This organic dust-induced inflammation could be explained by the fact that extracts of grain dusts can activate the complement cascade, via the alternative pathway (Edwards et al, 1976; Olenchock et al, 1980), as can wool dust (Donaldson et al, 1990). Generation of C5a could further amplify the inflammatory response since C5a causes macrophage secretion of neutrophil chemotaxins (Robbins et al, 1987).

Alveolar macrophages from endotoxin-treated rats could also contribute to inflammation by increased chemotactic activity (Christman et al, 1989). It was also reported that an initial exposure to endotoxin primed alveolar macrophages, resulting in an augmented response on subsequent challenges (Christman et al, 1989). Thus the organic dusts, used in the present study, which were contaminated with endotoxin, when deposited in the lung, could result in initial stimulation of macrophages. This could cause macrophage production of chemotaxins, and the generation of an acute inflammatory response enhanced by complement activation. If the dust persists in the lung, leachable products could further stimulate the macrophages, producing enhancement of the inflammatory response and stimulation of other cells by way of cytokines and other molecules.

Von Essen et al (1988) has suggested that exactly such a sequence of events occur in the lungs of grain-exposed workers. In addition Von Essen et al (1988) described the presence of neutrophil chemotaxins in the grain dust. In a previous study no evidence for a chemotactically active component of wool dust was evident (Donaldson et al, 1990). To our knowledge, there are no previous descriptions of aggregates of BAL cells following exposure to organic dusts.

4.3. Direct Injurious Effects of Dust Towards Epithelial Cells of the Lung and Alveolar Macrophages.

4.3.1. Epithelial Cells.

These cell types are the first cells likely to encounter depositing dust in the lung and therefore may be important participants in the initiation of an inflammatory response. Recurrent, persistent damage to the broncho-alveolar epithelium by dust could result in increased cough and production of mucus due to goblet cell hyperplasia. In addition, irritant receptors in the epithelium become more sensitive due to the dust. The role of endotoxin in epithelial injury is unknown, and the samples of dust and their leachates were shown to be heavily contaminated with endotoxin.

To study the effect of dust on epithelial cells of the lung, we devised a model which used a cell line derived from a human type 2 cell carcinoma (A549) (Lieber et al, 1976). The data obtained after treating epithelial cells with whole wool and grain dusts and products leached from the surface of the dust suggested that there was no appreciable injury towards these cells. Only the ledge sample of wool dust S caused significant lytic injury to A549 cells at the two highest concentrations. The

components of ledge dust could differ quite appreciably from airborne dust and so we consider these findings to be less relevant. They also explain our previous findings of toxicity of wool dust to epithelial cells (Donaldson et al, 1990) since the sample was ledge dust.

A number of environmental pollutants have been reported to affect alveolar epithelial cells. Oxidant gasses such as ozone (O₃), or nitrogen dioxide (NO₂), cause injury to airspace epithelial cells (Crapo et al, 1984; Evans, 1984). Other injurious agents such as mineral dusts and fibres also produce detrimental effects on epithelial cells (Donaldson et al, 1989; Donaldson et al, 1992a). Low concentrations of colophony fume, an organic product derived from pine resin and used in glues, varnishes and fluxes, caused detachment injury to A549 epithelial cells *in vitro* (Cullen et al, 1992). This may be related to the sensitization activities of colophony (Sherwood Burge, 1984).

A study close to the aim of the present one, which examined the effects of organic dusts and extractable products from the dust on epithelial cells was carried out by Ayars et al (1986). The study showed that aqueous extracts of cotton dust contained many biologically active compounds and that these were directly damaging to pneumocytes in terms of causing epithelial detachment from tracheal explants. Components identified in the extracts included tannin, a potent toxin towards epithelial cells of both the airspaces and airways. These effects were confirmed by *in vivo* experiments where tannins were instilled into rat lungs, producing desquamation and destruction of the bronchial epithelium.

Ayars et al (1986), corroborating our findings of the lack of direct injury to epithelial cells with endotoxin, found no correlation between the endotoxin content of dust and the amount of epithelial injury produced, even with large amounts of endotoxin (up to 50ug). The Ayars study concluded that it was tannin and other extractable compounds from cotton dust and not LPS, which contributed principally to the symptoms of chronic bronchitis in cotton workers. However, in other experiments, instilled endotoxin produced symptoms of chronic bronchitis in rabbits (Cavagna et al, 1969) but the effect on epithelial cells was not measured.

The absence of a direct toxic effect of dust on epithelial cells does not necessarily mean that epithelial compromise does not occur in dust-exposed lung. Indirect effects could be mediated through products released from macrophages and other resident cells triggered by the dust. Mediators released by activated macrophages

and neutrophils have been shown to be capable of damaging bystander cells such as epithelial cells (Donaldson et al, 1992a).

Measurement of direct toxic effects of a dust may represent a poor index of the potential of a dust to cause harm. Type 2 epithelial cells, can produce IL-8, which is a neutrophil and lymphocyte specific chemoattractant (Strieter et al, 1989a; Strieter et al, 1989b; Standiford et al, 1991). The cytokine may be important in our organic dust-exposed lung model but was not measured. The potent chemoattractant leukotriene B₄ may also be involved. This mediator, although not secreted directly by epithelial cells, can be generated from arachidonic acid, secreted by stimulated type 2 cells (Henderson, 1987). Thus although direct toxic effects of dust or leachates were not demonstrated, sublethal stimulatory effects on epithelial cells could lead to inflammation and should be the target of future investigation.

4.3.2. Alveolar Macrophages.

One important evolved function of the alveolar macrophage is phagocytosis of deposited particles, including bacteria (Holian and Scheule, 1990). In view of the large number of pro-inflammatory and immuno-stimulatory substances released by macrophages after phagocytic stimulation, these cells are likely to play a key role in the pulmonary response to wool and grain dusts. In addition lipopolysaccharide, a stimulator of macrophage secretion (Beutler et al, 1986; Feist et al, 1989), is present in the dusts. Although we later describe the stimulatory effect of organic dust on macrophages, we first addressed the question of whether organic dusts were overtly toxic to alveolar macrophages. We found no evidence of toxicity to macrophages by the grain or wool dust. As for epithelial cells, sub-lethal secretory effects could have been present and this is addressed below.

4.4. Stimulation of Macrophage Secretion by Organic Dusts.

As described above, the organic dust did not kill alveolar macrophages but the dusts could stimulate them. Macrophages have a wide range of secretory products (Rappolee and Werb, 1989) but the cytokines have been found to be particularly important in leading to inflammation and pathological change (Keshav et al, 1990). Tumor Necrosis Factor is member of the cytokine family and is produced in response to infection, inflammation or injury. This cytokine is produced by various cell types including macrophages/monocytes, lymphocytes, and vascular smooth muscle cells (Larrick and Kunkel, 1988; Warren, 1990). Secretion is triggered by stimuli such as interleukin-2, interferon-gamma, viruses and microorganisms. The

classical activator of TNF release is bacterial endotoxin (LPS) (Beutler et al, 1986; Feist et al, 1989) and so the presence of endotoxin in our organic dust samples strongly suggested that secretion of TNF could be important.

Treatment of rat alveolar macrophages with wool and grain dusts *in vitro* caused secretion of TNF in a dose dependent manner whilst untriggered macrophages produced negligible amounts of TNF. Wool and grain dust leachates, containing significant amounts of LPS, also caused production of TNF. The results of these experiments suggested that wool and grain dust depositing in the lung may have the potential to cause TNF release from macrophages and possibly other cells such as epithelial cells in the airspaces.

To further investigate the likely role of endotoxin in causing TNF release from alveolar macrophages *in vitro*, samples of dust leachate which were previously used were depleted of endotoxin by treatment with polymyxin. When these were used to stimulate rat alveolar macrophages, TNF content of supernatants was substantially reduced. This confirmed a major role for LPS in TNF production by alveolar macrophages treated with organic dust *in vitro*. By using reagent LPS, purified from *E. coli* bacteria, to produce supernatants, a clear LPS dose-dependent stimulation of TNF release was demonstrated.

The role of LPS in macrophage stimulation by wool dust was further investigated using peritoneal macrophages from mice with non-responder status to LPS stimulation (C3H/HEJ strain) or normal responders (C3H) (Adi et al, 1992). The macrophages from non-responder mice, when treated with organic dust, produced very low levels of TNF compared with the responder mice. In addition, leachates of dust which had been depleted of endotoxin produced an even smaller response. Taken together these findings strongly suggest that TNF secretion in the airspaces by alveolar macrophages could be a powerful pro-inflammatory stimulus in individuals inhaling organic dusts.

Beutler et al (1986) demonstrated the release of TNF by macrophages after endotoxin stimulation *in vitro*. In studies where LPS was administered to animals intratracheally, TNF levels in BAL were raised (Nelson et al, 1989). Interestingly, serum levels of TNF were not detectable, which suggests that TNF may be involved in compartmentalizing an inflammatory response. The production of TNF *in vivo* has been shown to occur as early as 3 hours after the inhalation of cotton dust (Ryan and Karol, 1991). Ryan and Karol (1991) also confirmed the presence of endotoxin in

substantial amounts in the cotton dust and suggested that this was the main stimulus for TNF release.

Endotoxin in association with organic dust has been extensively studied by Rylander and Lundholm (1978), who demonstrated that gram negative bacteria present on cotton dust and in the air of cotton mills could recruit increased numbers of inflammatory cells to the lung. The evidence that endotoxin contributes to chronic inflammation has been established by studies in which rabbits were exposed to endotoxin aerosols for several months (Pernis et al, 1961). Histological preparations showed the development of goblet cell hyperplasia and increased mucus secretion. These pathological changes could result from several distinct pathways involving mediator release, production of free radicals (not measured in the present study) and production of chemotaxins. Several other dusts have been shown to stimulate alveolar macrophage cytokine secretion in a manner similar to that shown by our organic dusts, including quartz (Driscoll and Maurer, 1991), asbestos (Donaldson et al, 1992b) and coalmine dust (Borm, 1988). Endotoxin is not implicated in the effect of these dusts, but they may have some endotoxin contamination.

In the airways, TNF production may be particularly significant. The airway epithelium may receive substantial exposure to depositing dust and/or endotoxin compared with the alveoli due to the mucus environment whose function is to trap and remove particulate material. Stimulation of macrophages in the airways by organic dust may produce localized increases in TNF, enhanced inflammation and, in the long term, bronchitis. An additional effect of TNF production in the airways could be the activation of cell surface adhesion molecules on leukocytes (Patarroyo and Makgoba, 1989) (see below).

For workers inhaling organic dusts such as wool and grain, symptoms of bronchitis and breathlessness may result from activation of macrophages in the alveolar region or the airways of the lung. Endotoxin associated with the dust may be a potent trigger for TNF release which then enhances and maintains the inflammatory response.

4.5. Adhesion Molecules.

The integrins are cell surface molecules which are important in interactions between leukocytes and the extracellular matrix (Kishimoto et al, 1989; Patarroyo and Makgoba, 1989; Aranout, 1990; Montefort and Holgate, 1991). These events depend on the ability of the adhesion molecules to become activated and deactivated.

Two of the most important molecules which mediate these cell interactions are ICAM-1 and LFA-1 (Diamond et al, 1990), and were first identified in "homotypic adhesion" assays where T and B-lymphocytes were stimulated to form aggregates of cells (Tamatani et al, 1991). Although these events do not occur in normal resting conditions, stimuli which activate intracellular signalling pathways can result in the activation of LFA-1, possibly through a phosphorylation reaction (Lauener et al, 1990). This type of aggregation event is blockable with monoclonal antibodies to LFA-1.

When adhesion molecules become up-regulated or activated and remain so, the result is likely to be inflammation and eventually pathological change. In the rat model following organic dust instillation, aggregates of cells were present in the lavage. These aggregates comprised only alveolar macrophages, with no lymphocytes. This argues against involvement of an immune component. We hypothesized that activation of cell adhesion receptors could be important in the aggregate formation in the short term and possibly in giant cell/granuloma formation in the long term.

By using an *in vitro* cell aggregation assay which we developed, we were able to demonstrate spontaneous formation of small aggregates of rat alveolar macrophages. In the presence of leukocyte triggers such as LPS and PMA, the number of cells making up each aggregate were dramatically increased demonstrating integrin activation. The classical leukocyte activators LPS and PMA were particularly potent in this regard.

In the presence of the organic dusts, the number of cells/aggregate were significantly increased. As described previously, rat alveolar macrophages produced TNF *in vitro* when treated with the organic dusts and TNF did significantly increase integrin activity, as measured in the assay. Supernatants from macrophages treated with dusts *in vitro* did not produce increased macrophage aggregation in our assay (table 3). Endotoxin was present in the dust leachates but macrophages treated with leachate produced aggregates of cells which were almost the same as the medium control group. Possibly, the concentrations of endotoxin may be central to this lack of increased aggregate size in the leachate treatment experiments. In the leachates, only nanogram amounts of LPS were present, the amount of LPS in the other experiments were in the microgram range. At concentrations of LPS up to and including 500ng/ml, no increase cell aggregation was shown. Activation of leukocytes can, however, result from stimulation with LPS at nanogram quantities, and this may be sufficient to prime the cells, after which aggregates of cells can form given appropriate stimulation.

An important component in the aggregation of macrophages is the CD14 receptor for LPS/lipopolysaccharide binding protein (LPS/LBP) (Lauener et al, 1990). LPS is bound by the serum protein LPS binding protein and this complex is recognized by CD14 (Schumann, 1992). The phagocytosis of bacteria and LPS-coated particles is then readily carried out.

Confirmation of the involvement of LFA-1 and ICAM-1 in the aggregation of macrophages produced by the organic dusts and was shown by using monoclonal antibodies against rat LFA-1 and ICAM-1 (Tamatani et al, 1991). Supernatant from hybridomas completely abolished aggregate formation when used undiluted. A clear dose response was demonstrated with anti LFA-1 alpha and beta and anti ICAM-1 antibodies. A separate study by Möst et al (1990), described a role for LFA-1 in the formation of giant cells. Initial aggregation of cells may be the starting point for this event. Rats treated with wool and grain dusts in our study, develop granulomata and giant cells after about one month post exposure.

The mechanism of aggregate formation involves several distinct requirements. Activation by PMA and LPS requires the presence of calcium and magnesium ions (Dransfield, 1990). Depletion of these divalent anions resulted in complete abolition of aggregate formation. Additionally, the protein kinase C inhibitor staurosporine reduces aggregate formation. The aggregation produced by the organic dusts was not reduced by staurosporine which suggests that the macrophage aggregation produced does not involve the PKC pathway of activation.

Taken together, these results, summarized in diagram 2 require some interpretation. *In vivo* aggregates are not formed unless there is a particulate stimulus ie wool dust particles - leachates had no aggregate-forming ability. *In vitro*, when spontaneous small aggregates occur these can be amplified by wool dust, LPS or PMA. This suggests that two signals are necessary - a first one that cause the nucleus of an aggregate to form and the second that cause it to enlarge. It appears that, *in vivo*, the first stimulus is a phagocytic one, the wool dust particles and the second is provided by LPS. *In vitro*, the spontaneous aggregation provides a nucleus on which LPS or PMA can stimulate accretion. These stages involve activation of adhesion molecules as shown by specific antibody blocking. The classical second messenger system of the cell is involved in this process in the case of LPS and PMA as shown by the abolishing effect of staurosporine. However, the enhancing effect of wool dust is not inhibited by staurosporine, suggesting that a different pathway, as yet inelucidated, is involved. In organic dust-exposed lung, up-regulation of

adhesion molecules could be involved in enhanced inflammation and the granuloma formation seen in wool dust-instilled lung (Möst et al, 1990).

Diagram 2.

<u>IN VIVO</u>	<u>AGGREGATES</u>
UNTREATED	-
WHOLE DUST	+++++
DUST LEACHATE	-
LPS	-
 <u>IN VITRO</u>	
SPONTANEOUS	+
WOOL DUST	++
GRAIN DUST	++
DUST LEACHATE	+
LPS	++++
PMA	++++
TNF	++
FIBRONECTIN	+

Our hypothesis was whether immune processing in the airways, represented by aggregates of mononuclear cells in our rat model could account for the symptoms of airway irritation and inflammation in the wool workers. The results demonstrate no involvement of lymphocytes precluding a classical immune interaction. Persistent up-regulation of adhesion molecules leading to interactions between cells of the lung could however, be of great potential importance in enhancing inflammation and tissue injury.

4.6. Lymph Node Lymphocyte Responses.

The demonstration that LPS in the organic dusts caused macrophage activation to release TNF, drew attention to the possible role of local immuno-stimulation in the draining lymph nodes following organic dust deposition. The lympho-stimulatory effects of LPS are well documented (Lei and Morrison, 1988) and LPS on/in dust could reach the lymph nodes; alternatively, activated alveolar macrophages could migrate there to cause stimulation of lymphocytes by continued cytokine release. A sequence of experiments revealed that instilled organic dust caused draining lymph node cell priming to proliferate in response to sub-optimal PHA.

In support of the likely role of LPS was the finding that washing the dust reduced its ability to cause immuno-stimulation when instilled, and the demonstration that

leachates of the dust, which contained LPS, could reproduce the local immunostimulation. Lymphocytes in culture did show increased thymidine uptake in response to LPS (data not shown) as widely reported. However, in contradiction to these results the instillation of LPS failed to elicit any lymph node stimulation. The most likely explanation for these results is that it is not LPS itself that translocates to the lymph nodes, but that alveolar macrophages, activated by LPS to release lympho-stimulatory cytokines migrate to the lymph nodes and cause the observed priming of the lymphocytes.

Previous evidence does suggest that macrophages carry particles to the draining lymph nodes (Harmsen et al, 1985). However, in a limited number of experiments (data not shown), we failed to demonstrate transfer of particles to the lymph nodes after instillation. Further work is clearly warranted in this interesting area of the role of non-specific immuno-stimulation in chronic organic dust-related pulmonary inflammation and the matter of transport of particles to the lymph nodes.

4.7. Prolonged Lung Inflammation and Granuloma Formation.

Instillation of wool and grain dust into rat lungs resulted initially in the generation of an acute inflammatory response with recruitment of neutrophils into the airspaces. This was a short-lived event and, by 3 days post-exposure, neutrophils were almost absent in the BAL. The histology from dust-instilled animals up to 3 months after instillation showed typical granulomata with multinucleated giant cells and mononuclear cell foci. Small airway walls were also involved and were thickened and fibrosed.

Granuloma formation in the lung results, for the most part, from initial exposure to a persistent stimulus and is a prominent feature of a variety of lung diseases (Kunkel et al, 1989). Granuloma are collections of inflammatory cells characterized mainly by the presence of mononuclear cells and which include epithelioid cells and multinucleated giant cells. In addition, in some types of granuloma, collections of lymphocytes, neutrophils and fibroblasts are often found around the periphery of the lesion (Garrett et al, 1984).

The type of granuloma formed depends mainly on the particle which is present. In the case of a non-antigenic particle or foreign body, the granuloma formed consists mainly of mononuclear phagocytes, and often there is no involvement of the classical immune system. In contrast, lesions which develop in response to antigenic stimuli such as fungal spores and organic material have, in association, enhancement of the

immune system. Lesions of this type result from conditions such as hypersensitivity pneumonitis and sarcoidosis, and are also associated with enhanced immunological effects. The lesions in these conditions are also found to be surrounded by collections of T-lymphocytes.

It is generally considered that granuloma formation initially requires the presence of an acute alveolitis (Hunninghake and Gadek, 1981), and this has already been demonstrated in our study. Patients with granulomatous lesions in their lungs, often show substantial numbers of lymphocytes in the BAL. In part of our wool study, aggregates of cells which we were able to recover from the BAL were almost entirely collections of macrophages, as characterized by immunostaining. However, the interesting question that arises is - are the cells making up the granulomata in the rat lung entirely mononuclear phagocytes, or is there immune involvement with the presence of T-lymphocytes. This question remains unanswered.

In a separate study, it has been shown that granuloma formation requires, or at least is promoted by, the presence of certain pro-inflammatory cytokines, in particular TNF and IL-1 (Kasahara et al, 1989). The study showed that agarose beads linked to TNF and IL-1 produced larger granulomatous lesions compared with beads linked to interferon-gamma or IL-2 when instilled intraperitoneally into mice. The present study has shown that rat alveolar macrophages release substantial amounts of TNF *in vitro* after treatment with whole wool or grain dusts and with leachates of the dusts. This supports the assertion that dust particles reaching the alveolar region of the lung initially provoke an acute inflammatory response, a suggested pre-requisite for granuloma formation (Hunninghake and Gadek, 1981). The persistence of the particle in the lung then initiates granuloma formation, possibly with some immune system involvement, in an attempt to "isolate" the particle. Endotoxin associated with the dust or leached from its surface is then able to trigger local secretion of TNF and possibly IL-1 from the resident mononuclear phagocytes. The increased concentration of cytokine may then promote and maintain the granulomatous lesion.

The progression to fibrosis after granuloma formation has been shown in several animal models of lung injury. In the wool and grain study, granulomas formed very soon after exposure to the dusts (Hunninghake and Gadek, 1981). Additionally, there was gradual progression to fibrosis, which was clearly shown around the periphery of airways. These events occurred after a single instillation of 1mg of dust, and it can therefore be assumed that there was some degree of persistent activation which initially caused the formation of a granulomatous lesion resulting in the derangement of the normal lung architecture, and finally to fibrosis. Persistent activation of

macrophages and inflammatory mediator release produced as the result of the failure of the lung to clear the dust could account for these changes. This is not to necessarily suggest that granuloma formation is a likely corollary of inhalation of wool or grain dust since the model we used here was rat instillation. It does however, underline the potent persistent biological activity of these particles that has been demonstrated throughout this study.

4.8. Summary of conclusions.

This study has shown that organic dust can produce recruitment of inflammatory cells, activation of alveolar macrophages, stimulation of immune cells and activation of cell adhesion molecules in a rat model of lung injury. In the toxicity experiments, no direct injury was evident toward alveolar epithelial cells or alveolar macrophages. This does not preclude sub-lethal effects where stimulation of the cells could occur resulting in activation and secretion of pro-inflammatory mediators. This was confirmed in in vitro experiments where organic dusts and dust leachates could produce secretion of TNF from alveolar macrophages. A large part of the cell stimulation was due to endotoxin present on the dusts and in the dust leachates, confirmed when endotoxin was depleted from the samples. The dusts also produced activation of cell adhesion molecules, manifest as homotypic aggregations of alveolar macrophages. Another important consequence of this could be increased adhesion of macrophages to epithelial cells leading to epithelial cell injury but this was not investigated. The possible involvement of the immune system was demonstrated in experiments where the lymph node lymphocytes from dust-treated rats were shown to be in a primed state. Priming could arise from mechanisms where activated macrophages could migrate from the lung to the lymph nodes and secrete pro-inflammatory cytokines, particularly TNF, although other molecules may be important. These events, singly or in concert could provide the starting point for inflammation and for changes in lung function and pathology, which may be relevant in workers exposed to organic dust.

4.9. Further work.

The study has pointed out several areas where further work would illuminate the results obtained in the present study.

1. General.

All the results of the present study were obtained after intratracheal instillations. What are the effects in inhalation experiments?

2. Aggregation.

What is the secretory potential of cell aggregates and are they adhesive to lung epithelial cells? This could have important consequences for epithelial cell injury/stimulation and further stimulation of alveolar macrophages.

3. Role of Cytokines.

Are cytokines such as TNF involved in lymph node stimulation? Although epithelial injury by dust was not demonstrated the dusts could cause stimulation of release of pro-inflammatory mediators (such as cytokines) by the epithelial cells.

4. Mechanisms and Consequences of the lymph node stimulation.

What are the consequences of the observed lymph node lymphocyte priming? In particular, are pro-inflammatory lymphokines produced in the lymph nodes of organic dust-treated lungs? Are these effects mediated by activated macrophages migrating to the nodes where they could cause lymphocyte activation through the release of cytokines? What is the role of endotoxin in these events and could the effects be due to organic dust particles reaching the lymph nodes?

5. Complement System.

What role does the complement system play in these events? Previous work with wool and grain dusts has demonstrated potent complement-activating activity. This was not addressed in this study but the role of complement needs to be incorporated into any complete hypotheses in the inflammatory effects of organic dusts.

6. Granuloma Formation.

Are the granulomata the result of a foreign body or immunological effect; and would they be produced with inhalation or are they an artefact of instillation?

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